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CALLOSE DEPOSITION AND ALUMINUM RESISTANCE IN WHEAT

BY

KYMBERLY ANNE SCHREINER

A thesis submitted to the
Faculty of Graduate Studies and Research in partial fulfillment of the
requirements for the degree of MASTER OF SCIENCE.

IN

PLANT PHYSIOLOGY

DEPARTMENT OF BOTANY

Edmonton, Alberta

FALL 1992

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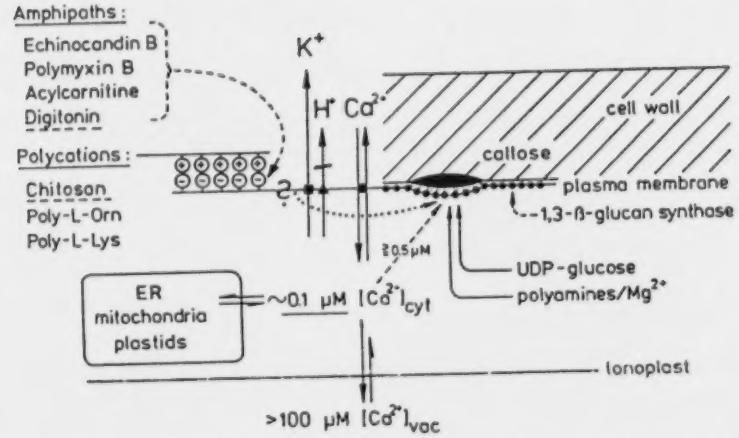

Fig. 2. Synoptic view of some reactions presumably involved in the induction of callose deposition. Polycations or certain amphipaths appear to perturb the plasma membrane, and trigger in an unknown manner (indicated by ?) an increase in K^+ efflux and an external alkalinization which is possibly due to a decrease in H^+ export. The associated net Ca^{2+} uptake into the cell may increase the concentration of free Ca^{2+} in the cytoplasm from the resting level, normally held at about 0.1 µM by pumping Ca^{2+} into the vacuole, organelles, and the extracellular space, to a range above 0.5 µM, thus activating the 1,3-ß-glucan synthase. This enzyme is in vitro strictly dependent on Ca^{2+}, and gains increased sensitivity toward Ca^{2+} by the presence of 200 µM spermine or 4 mM Mg^{2+}. Additional effectors may cooperate in vivo with Ca^{2+} (*dotted line*, for details see text). The events (depicted *above*, side by side) should be imagined to take place in the same cell surface region to explain the often very localized callose deposition. Note that conditions leading to callose synthesis can also induce phytoalexin synthesis, suggesting that Ca^{2+} is also one of the second messengers in this process (After Kauss 1987a)

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Intact cell:

UDP-Glc —(Plasma-membrane-localized glucosyltransferase ~ 18 kd Complex; low Ca^{2+}; negative $\Delta\Psi$)→ cellulose

Intact cell plus DCB:

18 Kd ~ DCB dissociated or nonfunctional

UDP-Glc —(Plasma-membrane-localized glucosyltransferase; low Ca^{2+}; negative $\Delta\Psi$)→ no product

Damaged cell or isolated membranes:

18 Kd dissociated, found in soluble fraction

UDP-Glc —(Plasma-membrane-localized glucosyltransferase; elevated Ca^{2+}; dissipated $\Delta\Psi$)→ callose

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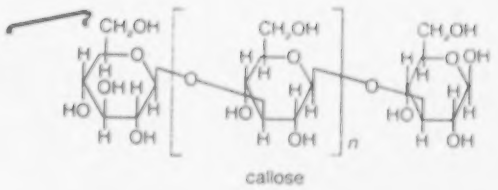

Figure 8-19 The molecular structure of callose. Note the interesting 1,3-linkage between β-D-glucopyranose residues, which causes a tight coiling of the molecular callose chain.

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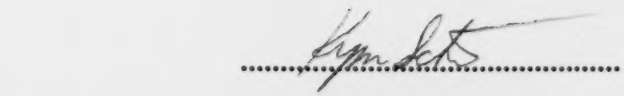

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Gregory J. Taylor

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Abstract

Soil acidity is a major growth-limiting factor in crop production. This growth limitation is primarily due to aluminum (Al) toxicity. Yield losses may be reduced by screening crops for Al-resistance using an early stress response as a selection criterion. One selection criterion uses callose deposition as an early, sensitive marker for membrane injury. This study examined the relationship between callose deposition and Al-resistance in *Triticum aestivum* L. Callose was quantified and localized in the roots and leaves of three-day old seedlings of Al-resistant lines, 'Atlas-66' and PT741, and Al-sensitive lines, 'Scout-66' and 'Katepwa', which were grown for forty hours in solution culture containing Al (0 - 1000 μM). Root length and root callose content were better indicators of Al resistance than leaf length and leaf callose content. Leaf length was variable after Al treatment. Leaf callose content was at or near the minimum level of detection of the spectrofluorometer used. Root length was more sensitive to Al treatment than root callose content, however, differences between Al-resistant and Al-sensitive lines were larger with root callose content.

The content and localization of callose in Al-treated plants suggested that Al caused little damage to the membranes in leaves, but did much damage in roots of Al-sensitive lines. Root callose content increased by as much as 1100% in 'Katepwa' at 1000 μM Al. The pattern of callose deposition was similar to reports of Al distribution. Large deposits of callose were located in the root cortex and root cap of Al-sensitive lines. Callose presence was confirmed in freeze-substituted tissues by reduced fluorescence after treatment with callose inhibitor, 2-deoxy-D-glucose, or with callose hydrolase.

These studies suggested that Al resistance may occur at the root by preventing Al uptake, although further investigation is needed. Callose studies may be useful as a non-destructive screen for Al-resistance, and as a marker for the transport of toxic Al species, especially where growth responses are difficult to determine.

Acknowledgements

I am grateful to my supervisors, Dr. John Hoddinott and Dr. Gregory J. Taylor, for their patience and support throughout my thesis. Thanks also to Dr. D. D. Cass (Botany) and Dr. L. Wang (Zoology) for instruction and use of lab equipment, and to Dr. H. Manning (Chemistry) for providing important chemicals. Finally, I gratefully acknowledge the encouragement I received during this time from my family and husband. This work was supported by the Department of Botany, the University of Alberta Faculty of Graduate Studies and Research, and an NSERC strategic grant awarded to my supervisors and Dr. K. G. Briggs (Plant Science).

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1. Introduction

We must be wary of falling into the all too common trap of viewing the plant as a green animal.

Gilroy & Trewavas 1990

Land plants, unlike animals, have no means to move to other locales: survival depends on their ability to resist the stresses around them. These stresses may include adverse weather, poor soil, insects, and disease. In fact, according to Levitt (1980), any environmental factor capable of inducing a potentially injurious strain (physical or chemical change) in living organisms is a "stress". Stress-induced change occurs in three steps: perception of a stimulus or stress, transduction of a message to other plant parts, and physiological response. A plant that either fails to respond to a potential stress, or does so in a way which promotes survival, is considered stress-resistant. Stress resistance is of much interest to plant scientists, including breeders 'designing' plants to survive stressful environments, and physiologists seeking the basis of stress resistance.

Perhaps the most logical site for studies of stress resistance is the plant plasma membrane, as it: (i) is the first living part of the plant in contact with the stress, (ii) perceives and responds to changing environmental conditions, and (iii) bears enzymes that are sensitive, early markers of plant stress. The present study examines the content and localization of callose deposits in differentially resistant lines of *Triticum aestivum* after aluminum (Al) treatment. As outlined in the following sections, Al is the primary growth limiting factor for plants grown on acidic soils, and callose is one of the most sensitive markers of injury at the plant membrane.

1.1. Aluminum Stress on Acid Soils

Soil acidity is a major growth limiting factor for plants in many parts of the world (Foy 1983, Foy *et al.* 1978). Nearly 40% of the world's arable soils and up to 70% of the lands suitable for food and biomass production are acidic (Haug 1984, Foy *et al.* 1978; Figure 1-1). Although low pH soil may occur naturally, its frequency is increasing due to agricultural, manufacturing, mining, and waste disposal practices (Foy *et al.* 1978). For example, frequent use of acid-forming nitrogenous fertilizers can exacerbate the pH problem (Foy *et al.* 1978). In Canada, soil acidity is an agricultural problem in every province except Manitoba (Hedlin & Kraft 1984).

Figure 1-1: Global distribution of acid soils (///) and major areas of wheat production (\\\). Areas of overlap are shaded in black. (Reproduced, with permission, from the Ph.D. thesis of S. Macfie, 1991).

Under acidic soil conditions, it is Al that primarily limits plant growth (Baligar *et al.* 1991). Aluminum is a key constituent in most common rocks (except limestone and sandstone) and is the most abundant metal in the crust of the earth (Haug 1984). Despite its abundance, only soluble forms of Al affect plants. Aluminum tends to accumulate in

plant roots, although high concentrations of Al also occur in the leaves of several perennial species including tea (*Thea sinensis* or *Camellia sinensis*) and some Proteaceae (Chenery & Sporne 1976). In fact, Al, is present in everything from carrots to milk, and contributes between 9 and 14 mg of Al to the human diet - far in excess of the recommended 3 mg daily maximum (Cox 1991).

The prevalence of this metal is of concern. Aluminum is toxic to fish and other water biota, and is recognized as a neurotoxin (Cox 1991). It affects learning and memory performances of animals and has been linked with Alzheimer's disease in humans (McLachlan *et al.* 1991). Lowering the Al content of food is one means of reducing Al exposure. Plants are a logical choice for such an effort. Not only are plants the link between soluble inorganic Al and animals higher up the food chain, but some plants may prevent or impede the accumulation of this metal in their tissues (Taylor 1991). Alternatively, some plants may accumulate Al and thereby promote the survival of neighbouring plants. Understanding the mechanisms of Al resistance may aid breeders in developing Al-resistant plants to increase crop production on acidic soils, to detoxify metal-contaminated soils, and to lower the Al content of food.

1.1.1. Physiological Plant Responses

Locating the mechanisms of Al resistance is aided by an understanding of where and how soluble Al affects the plant. Since several reviews exist on the physiological responses of plants to Al (for example Foy *et al.* 1978, Haug 1984, Taylor 1991) only a few aspects will be discussed here.

Aluminum is primarily rhizotoxic. Most of the symptoms of Al toxicity occur at the root level, where Al first interacts with the plant. Aluminum tends to destabilize the plasma membrane by displacing Ca^{2+} and other divalent cations (Akeson *et al.* 1989, Caldwell 1989) and by binding to the phosphate group of phospholipids to decrease lipid membrane fluidity (Matsumoto 1988). These interactions with Al cause

membranes to become more permeable to nonelectrolytes (Chen *et al.* 1991) and less permeable to water (Zhao *et al.* 1987) and nutrients (Foy & Burns 1964), especially phosphorous, potassium and calcium (Foy *et al.* 1978). As a result, Al action at the root plasma membrane may ultimately lead to whole-plant responses, including root growth inhibition, drought susceptibility, and nutrient deficiencies (Foy 1983).

1.1.2. Preventing Aluminum Toxicity

Whether or not plants on acidic soils exhibit Al toxicity symptoms depends largely upon the type and activity of the Al species present in the soil, and the resistance of the plant species (Marschner 1991). The Al species responsible for plant toxicity are still in question, however, two polyvalent cations that are particularly toxic under laboratory conditions are Al_{13}^{7+} and Al^{3+} (Kinraide & Ryan 1991). Maintaining a soil pH that is slightly basic or slightly acidic may alleviate soil toxicity symptoms but may not always be practical. Amelioration of strongly acidic soils is costly, especially where fuel and lime are scarce (Foy 1983). In addition, applied compounds like calcium may readily leach down through the soil profile and become unavailable (Ritchey *et al.* 1989). Finally, concern over energy conservation and environmental pollution has made agriculturalists reconsider "changing the soil to fit the plant" (Foy 1983). It may be more practical to 'fit' the plant to a particular soil.

Aluminum toxicity also depends upon the plants themselves. Aluminum resistance is an heritable trait (Foy 1988) which may be selected for in breeding programs. Although screening for Al-resistance is time-consuming and costly, this process may be improved if plant resistance to this metal is better understood. Resistance may occur by two strategies: tolerance and avoidance (Levitt 1980). As the term implies, a plant showing tolerance actually 'tolerates' the entry of a stress into its symplasm and survives by minimizing any injury. For example, Al may be exported from the cytoplasm, compartmentalized into cell vacuoles, inactivated by cytosolic

chelators or Al-binding proteins, or tolerated by the evolution of Al-tolerant enzymes or by a general reduction of enzyme activity (Taylor 1991). A plant showing avoidance 'avoids' the entry of a stress by partially or completely excluding it (Levitt 1980). In this case, the activity of Al of the surface of the plasma membrane is reduced by adsorption of Al to the cell wall, exudation of chelate ligands or phosphate, or precipitation of $Al(OH)_3$ by the rhizosphere pH (Taylor 1991).

1.2. Callose - A Marker for Membrane Injury

Whether tolerance, avoidance, or both is responsible for Al-resistance in plants, it may be argued that the most logical site for investigating Al-resistance is at the root plasma membrane. The plasma membrane is the first living part of the plant in contact with the stress, and is one of the first parts of the plant to respond. Further, the membrane bears several sensitive marker enzymes which are induced by plant injury. In fact, enzymatic changes in plants may be used to evaluate the potential phytotoxiciy of metal-contaminated soils (van Assche & Clijsters 1990). Low levels of metal-induced changes in enzyme activity are detectable before toxicity symptoms are observed (van Assche & Clijsters 1990). Any attempt to move the discriminatory criteria closer to the initial event of stimulus perception would be expected to increase the selectivity of procedures used (Bennet & Breen 1991).

One suitably 'close' criterion is callose deposition after stress perception. Callose deposition is one of the most rapid and common plant responses to stress. Callose consists primarily of β-1,3 linked glucose molecules (Figure 1-2) which form a tightly coiled and unbranched helix (Sathyanarayana & Rao 1971). Frequently, compounds such as lignin and phenol precursors embed in this matrix (Mueller & Beckman 1988). This helix is permeable to triphenylmethane dyes, such as water-

soluble aniline blue, which fluoresce an intense yellow in the presence of these glucose polymers and ultraviolet excitation light (Nakamura *et al.* 1984). Callose is primarily identified by its fluorescence, but also by its insolubility in water and methyl sulfide, or its solubility - with difficulty - after isolation in alkali (Clark & Villemez 1972).

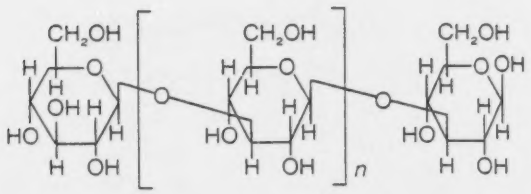

Figure 1-2: Molecular structure of callose. The 1,3 linkage causes a tight coiling of the molecular chain. (Reproduced, with permission, from Salisbury & Ross 1992 in Plant Physiology, 4th ed., © 1992 by Nelson Canada.).

Callose was observed as early as 1867 by Nägeli (in Currier 1957). Since then, callose has been studied in a wide range of plant processes, including plant development, growth and regulation, and cellular and whole plant transport (Table 1-1). Callose is also studied in stress physiology, including biological, physical and chemical stresses (Table 1-2). As suggested by the number of stresses which induce callose deposition, it is a general stress response. In fact, callose is associated with two other general stress-related compounds: ethylene and chitinase (Jaffe 1984).

Until recently, chemically-induced callose deposition has received little attention. Studies of chemical stresses include glutaraldehyde fixation (Hughes & Gunning 1980), boron toxicity (McNairn & Currier 1965) and deficiency (van de Venter & Currier 1977), and cobalt, nickel and zinc (Peterson & Rauser 1979), ozone (Fink 1991), and manganese toxicities (Wissemeier & Horst 1987, 1988). Of particular relevance to this thesis are recent studies of Al toxicity and callose deposition.

Wissemeier *et al.* (1987) found that root callose deposition in soybean (*Glycine max* L.) was a more sensitive indicator of Al injury than was root elongation. Schaeffer & Walton (1990) found that callose deposition after treatment with 200 μM Al was higher in mesophyll cell protoplasts isolated from Al-sensitive lines of *Avena sativa* than in protoplasts isolated from Al-resistant lines, although this was not true for *Hordeum* sp. Jorns *et al.* (1991) found that *Picea abies* seedlings deposited callose at the periphery of their roots within 3 hours of treatment with 170 μM Al. In none of these studies was callose deposition associated with Al resistance.

Table 1-1: Examples of the involvement of callose in plant processes.

PROCESS	SPECIES	REFERENCE
STRUCTURE & DEVELOPMENT		
Cell shape	angiosperms	Pooviah 1974
Sporocyte wall	angiosperms	Heslop-Harrison 1966
Cell wall	*Phaseolus vulgaris*	Northcote *et al.* 1989
Sieve pore	dicots	Klein *et al.* 1991
	Gossypium hirsutum	Waterkeyn 1981
Pollen grain	*Lilium henryi*	Esau & Thorsch 1985
	Triticum aestivum	Eleftheriou 1990
Root hair	angiosperms	Heslop-Harrison 1966
	Zea mays	Clarke & McCully 1985
Pollen tube rejection	*Secale cereale*	Vithanage *et al.* 1980
	Brassica oleracea	Singh & Paolillo, Jr. 1990
Fruit development	*Lycoperisicon esculentum*	Ma *et al.* 1991
GROWTH & REGULATION		
Leaf abscission	*Citrus sinensis*	Jaffe & Goren 1988
Gravitropism	*Zea mays, Pisum sativum*	Jaffe & Leopold 1984
Seed dormancy	*Trifolium subterraneum*	Bhalla & Slatterly 1983
	Sesbania punicea	Bevilacqua *et al.* 1987
Growth regulators	*P. vulgaris*	Abeles & Forrence 1970
Cellulase activity	*Acetobacter xylinum*	Delmer 1987
TRANSPORT		
Blockage of phloem	*Vitis vinifera*	McNairn & Currier 1965
& vascular tissue	*V. vinifera*	Aloni *et al.* 1989
Diffusion barrier	*Nicotiana glutinosa*	Schuster & Flemming 1976

Table 1-2: Examples of the involvement of callose in biological, physical, and chemical plant stresses.

STRESS	SPECIES	REFERENCE
BIOLOGICAL		
Fungi	*Brassica oleracea*	Aist 1977
	Hordeum sp.	Skou *et al.* 1984
Viruses	*Phaseolus* sp.	Wu *et al.* 1969, Hiruki & Tu 1972
Bacteria	*Catharanthus roseus*	Kauss *et al.* 1991
PHYSICAL		
Mechanical	*Prunus cerasus*	Dekazos & Worley 1967
Ultrasound	*Gossypium hirsutum*	Currier & Webster 1964
Plasmolysis	*Elodea, Allium cepa*	Prat & Roland 1971
Temperature	*Phaseolus* sp.	Clark & Villemez 1972
	crop varieties	Majumder & Leopold 1967
Light	*Datura stramonium*	Conti *et al.* 1983
CHEMICAL		
Fixation	dicots	Hughes & Gunning 1980
Ozone	*Picea abies*	Fink 1991
B	*Elodea*	Currier 1957
	Phaseolus vulgaris	McNairn & Currier 1965
	P. vulgaris, G. hirsutum	van de Venter & Currier 1977
Co, Ni, Zn	*P. vulgaris*	Peterson & Rauser 1979
Mn	*V. unguiculata*	Wissemeier & Horst 1987, 1988
Al	*Glycine max*	Wissemeier *et al.* 1987, Horst *et al.* 1991, Schaeffer & Walton 1990
	cereals	
	Picea abies	Jorns *et al.* 1991

1.2.1. A Model of Callose Deposition

Proper interpretation of these experimental results requires a knowledge of callose deposition. Critical to this process is the enzyme (EC 2.4.1.34) that acts during the transduction step to synthesize callose. This enzyme has been referred to in the literature as: callose synthase, glycosyltransferase UDP-glucose: (1,3)-β-glucan synthase, glucan endo-1,3-β-glucosidase, and glucan synthase II. This thesis simply refers to the enzyme as callose synthase.

Three forms of callose synthase are distinguishable by their location and mode of induction (van den Bulcke *et al.* 1989). The first form occurs in coated vesicles and has limited activity (Payne *et al.* 1990). The second "basic" form is localized in the cell vacuole and is induced by pathogens or ethylene treatment. Its constitutive level of expression is under developmental control in young cells (van den Bulcke *et al.* 1989). The third "acidic" form occurs along the plasma membrane and may synthesize both cellulose and callose, but never simultaneously (Delmer 1987; Figure 1-3). It is this latter form of callose synthase that is referred to for the remainder of this thesis.

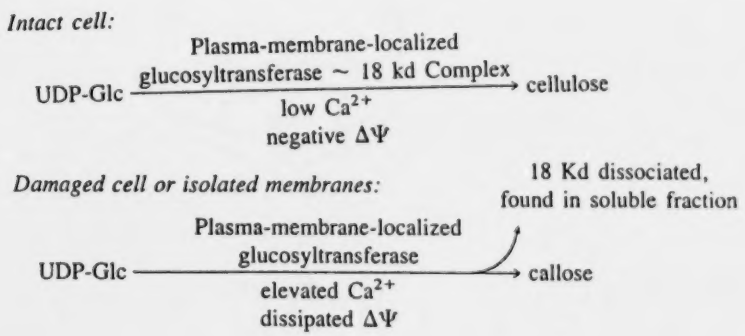

Figure 1-3: Model for the regulation of callose and cellulose synthesis. On the top line, intact cells with low Ca^{2+} levels favour the association of an 18 kd polypeptide with the enzyme glucosyltransferase (callose synthase), resulting in cellulose production. On the bottom line, damaged or isolated membranes with high Ca^{2+} levels and localized disruption in membrane electrical potential favour the production of callose. Abbreviations: (UDP) uridine diphosphate; (Glc) glucose. (Adapted, with permission, from Delmer 1987 in the Annual Review of Plant Physiology, Vol. 38, © 1987 by Annual Reviews Inc.).

Callose deposition occurs rapidly, within minutes of mechanical wounding. Such a rapid response suggests that *de novo* synthesis of the enzyme cannot account for stimulation of its activity (Kohle *et al.* 1985). Rather, callose synthase may be activated primarily by Ca^{2+} influx (Kauss 1986, Kohle *et al.* 1985) through transport channels in the plasma membrane (Kauss 1986; Figure 1-4). Recent evidence suggests

that decreases in cytoplasmic pH cause the release of vacuolar forms of β-furfuryl-β-glucoside, which may act as a second regulator of callose synthase (Ohana *et al.* 1992, Atkinson *et al.* 1990). Intracellular messengers like Mg^{2+}, polyamino acids, and spermine may also have regulatory control by influencing Ca^{2+} influx (Kauss & Jeblick 1986).

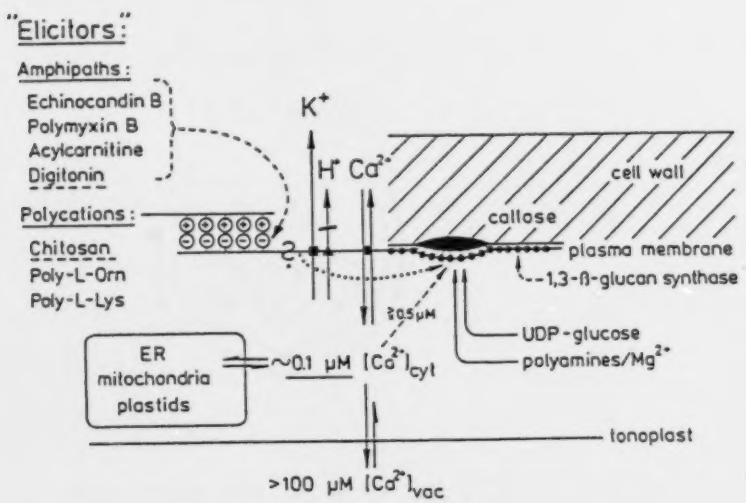

Figure 1-4: Model of callose deposition. Polycations or certain amphipaths may perturb the plasma membrane and trigger, in an unknown manner (indicated by ?), an increase in K^+ efflux and an external alkalinization is possibly due to a decrease in H^+ export. The associated net Ca^{2+} uptake into the cell may increase the concentration of free Ca^{2+} in the cytoplasm from the resting level, normally held at about 0.1 µM by pumping Ca^{2+} into the vacuole, organelles, and the extracellular space, to a range above 0.5 µM, thus activating the 1,3-β-glucan synthase (callose synthase). *In vitro* this enzyme is strictly dependent on Ca^{2+}, and gains increased sensitivity toward Ca^{2+} by the presence of 200 µM spermine or 4 mM Mg^{2+}. Additional effectors may cooperate *in vivo* with Ca^{2+} (*dotted line*). These events could explain the often very localized callose deposition. Note that conditions leading to callose synthesis can also induce phytoalexin synthesis, suggesting that Ca^{2+} is also one of the second messengers in this process. (Reproduced, with permission, from Kauss 1990 in The Plant Plasma Membrane, © 1990 by Springer-Verlag Heidelberg).

The intervening steps between enzyme induction and callose deposition are less well-known. Studies using antibodies specific for 1,3-β linkages suggest that the Golgi apparatus and its vesicles are not involved, as neither contain any callose (Northcote *et al.* 1989). Rather, the plasma membrane, in conjunction with the endoplasmic reticulum, may deposit callose extracellularly. Alternatively, since callose synthase may be a transmembrane enzyme, callose may be deposited during its synthesis.

1.2.2. Physiological Role of Stress Callose

Several facts suggest that callose may have some physiological role in the plant cell. Firstly, energy is expended to regulate callose deposition. Secondly, both callose and its enzyme are conserved in several other organisms. Population geneticists argue that conservation usually occurs by selection, especially if the trait requires an input of energy. Homologs of this higher plant compound occur in brown seaweed, algae, fungi, and yeast (Wu *et al.* 1991). The polypeptide composition and subunit structure of callose synthase is similar in many plants (Wu *et al.* 1991). Thirdly, callose deposition is an extremely common response as demonstrated in Tables 1-1 and 1-2.

Among the roles suggested for callose are: wound-healing, preventing cytoplasm expulsion and excessive loss of ions, regulating passive membrane transport, and mediating the symplastic movement and intercellular communication between cells of a plant. These proposed roles assume that sufficient callose is deposited at the intercellular connections to seal adjacent cells (Figure 1-5).

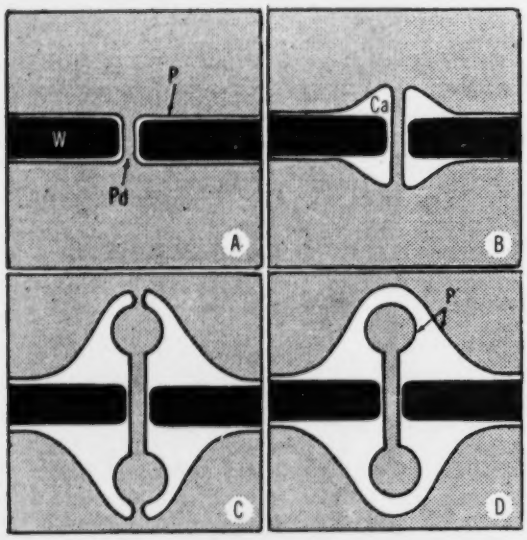

Figure 1-5: Model for the blocking of intercellular connections by callose. A) normal plasmodesma. B-D) progressive deposition of callose. Abbreviations: (Pd) plasmodesma, (Ca) callose, (W) primary cell wall; (P) plasma membrane. (Reproduced, with permission, from Allison & Shalla 1974, Phytopathology, Vol. 64, © 1974 by American Phytopathology Society).

1.3. Overview

This research began with hopes of finding evidence for a physiological role of callose, in particular, the possibility that callose may impede the entry or transport of Al into wheat plants. As discussed earlier, Al toxicity is a global problem and has been shown to induce callose synthesis in several plant species. Wheat was chosen as the experimental species in this study because of its agricultural importance in countries having acid soils (see Figure 1-1), and its known differential resistance to Al. The winter wheat cultivars 'Atlas-66' and 'Scout-66' are international standards for Al-resistance and Al-sensitivity, respectively. The spring wheat lines PT741 (an experimental line developed by Dr. K. G. Briggs, Plant Science, University of Alberta) and 'Katepwa' are Al-resistant and Al-sensitive, respectively. The following questions were examined: (i) is callose deposition associated with Al resistance in wheat, (ii) do root and leaf tissues of differentially Al-resistant lines respond to Al, and (iii) does the

localization of callose support what is known about Al transport and distribution. To address these questions, callose from roots and leaves were quantified using a spectrofluorometer and localized by fluorescence microscopy, as outlined in the following two chapters.

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2. Quantification of Callose in Aluminum-Treated Wheat[1]

2.1. Introduction

Callose, a glucose polymer, is deposited in response to a variety of stresses and developmental processes. As a general stress response, callose synthesis is induced when plasma membrane disturbances cause a localized influx of calcium ions (Kauss 1990) and release vacuolar forms of β-furfuryl-β–glucoside (Ohana *et al.* 1992). This cation and β-glucoside bind to separate sites on the cytoplasmic side of the membrane-bound callose synthase (Ohana *et al.* 1992, Fredrikson & Larsson 1989), and induce the extracellular deposition of callose, primarily at the sieve plates and plasmodesmata. The locations of these deposits have led to the hypothesis that callose may act as a valve regulating the passage of compounds between cells (Crafts & Currier 1963).

The proposed regulatory role of callose has been studied for a number of stresses, with conflicting results. Callose did not reduce carbon translocation in response to excess cobalt, nickel and zinc (Peterson & Rauser 1979), nor to boron deficiency (van de Venter & Currier 1977) in *Phaseolus vulgaris* and *Gossypium hirsutum*; however, it may have prevented the spread of Potato Virus M in the primary leaves of *P. vulgaris* (Hiruki & Tu 1972). Callose presence, alone, does not support a regulatory role in stress resistance. Callose must be synthesized at the proper time and place, and in sufficient quantity to be effective (Faulkner & Kimmins 1978).

The present study quantified callose deposition in *Triticum aestivum* after aluminum (Al) stress. Several studies suggest that callose may be useful as a physiological marker for Al injury (Wissemeier *et al.* 1987, Jorns *et al.* 1991), however, evidence for callose involvement in Al resistance is lacking. Callose deposition in the

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[1]A version of this chapter is being prepared for publication.

mesophyll cell protoplasts of various cereals was not associated with Al resistance at the whole plant level (Schaeffer & Walton 1990). Aluminum-resistant and Al-sensitive mesophyll cell protoplasts of *Avena sativa* had more callose deposits after Al treatment; however, preventing callose deposition with cycloheximide did not result in cell death (Schaeffer and Walton 1990). Further investigation of callose involvement in Al resistance is warranted because callose observed by fluorescence may inaccurately estimate callose content, callose is not specifically inhibited by cycloheximide, and responses in leaf protoplasts may not reflect those at the whole plant level.

To my knowledge, this study is the first to quantify callose deposition after Al treatment at the whole plant level. Results indicated that callose deposition in the roots and leaves of wheat was not positively correlated with Al resistance. Large differences in root callose content were, however, observed between Al-resistant and Al-sensitive lines. Callose quantification could be useful in short-term physiological studies of membrane injury or as a non-destructive screen for Al resistance in whole plants or cell suspension cultures.

2.2. Materials and Methods

2.2.1. Plant Lines and Growth Conditions

Seeds of two Al-resistant wheat (*Triticum aestivum* L.) lines ('Atlas-66' and PT741) and two Al-sensitive lines ('Scout-66' and 'Katepwa') were surface-sterilized in 1.2% sodium hypochlorite for 20 minutes and germinated overnight in an aerated 0.005 $g \cdot L^{-1}$ solution of the fungicide Vitavax. Seeds were set on nylon mesh suspended on 10 L of aerated nutrient solution containing ($\mu mol \cdot L^{-1}$): NO_3-N (3300), NH_3 (300), P (100), K (800), Ca (1000), Mg (300), S (100), Cl (34), Na (20.2), Fe (10), EDTA (10), B (6), Mn (2), Cu (34), Zn (0.5), and Mo (0.10) at pH 4.50.

Plants were grown in a controlled environment chamber at 24°C with 16 hours of light (97% relative humidity), at a light intensity of 315 $\mu mole \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation at plant base level. Although not controlled, solution temperature was 20 ± 1°C. After 1 day of growth, nine uniform seedlings were transferred to each of seventeen beakers containing aerated nutrient solution and one of 17 different Al treatments ($AlK(SO_4)_2 \cdot 12H_2O$; 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 400, 700, 1000 $\mu mole \cdot L^{-1}$). Plants were placed on a nylon disc floating on a strip of Styrofoam for added buoyancy. The experiment used a randomized block design with seventeen levels of Al and four wheat lines. Three replications were blocked in time, with overlapping growth conditions, giving a total of 204 experimental units.

2.2.2. General Plant Morphology and Organ Lengths

For each beaker, the general plant morphology, mean maximum root length, and mean leaf length of five uniform seedlings were recorded at time of harvest. Maximum root length was determined for the longest seminal root; leaf length was determined from seed emergence to the tip of the oldest blade. Primary growth data were untransformed and analyzed by General Linear Model and Analysis of Variance procedures available on the SAS (version 6.03; SAS 1989). Statistical significance was defined at the 0.05 probability level.

2.2.3. Determination of Callose Content

The following procedures were adapted from Bonhoff & Grisebach (1987) and Kohle *et al.* (1985). Approximately 85 mg fresh weight of root tissue or 110 mg of leaf tissue was excised and soaked for 60 minutes in 2 mL of ethanol (95%), containing 0.1 mM 2-deoxy-D-glucose (DDG, a callose synthase inhibitor), to remove soluble autofluorescent material. Adding DDG to the extraction medium yielded 6% less callose deposition than did a DDG-free ethanol soak. After draining the ethanol,

callose was released into solution by homogenizing the tissue with a Brinkmann Homogenizer in 1 mL NaOH (1 M). Another 1.25 mL of NaOH was added to the homogenate by rinsing the homogenizer blades. After heating for 15 minutes at 80°C, the solubilized callose was separated from cell debris by centrifugation for 5 minutes at 380 *g*.

Callose content was determined from a dilution of the supernatant by adding 100, 200, or 400 µL of the supernatant plus enough NaOH (1 M) for a total volume of 400 µL. These solutions were mixed with 800 µL of 0.1% [w/v] Aniline Blue (water soluble, C.I. 42755, lot # 60894; PolySciences, Warrington, PA, USA), a fluorescent stain for callose which produces a violet-red colour. The pH was adjusted to a neutral or slightly acidic pH by adding 420 µL of 1 M HCl and 1180 µL of 1 M glycine/NaOH buffer (in 1 M NaOH, pH 9.5), causing the solution to change to a deep blue colour. Solutions were thoroughly mixed. After standing for 20 minutes in a water bath at 50°C, and for 30 minutes at room temperature (20°C), the aniline blue became almost completely decolourized.

Fluorescence of the assay was measured by a Perkin-Elmer spectrofluorometer (excitation 398 nm, emission 495 nm). Peak excitation and emission wavelengths of pachyman standards were determined by spectrum analysis using an SLM Aminco 8000C spectrofluorometer (slit 4 nm, energy *hv* 700 J·cycle). A standard curve was made using a freshly prepared solution of β-1,3 glucan pachyman from *Poria coccus* (lot # 902569, Calbiochem, San Diego, CA, USA) in 1M NaOH. Pachyman has a β-1,3 linked glucose backbone associated with a variable, but low, proportion of internal β–1,4 or β–1,6 linkages (Verma *et al.* 1982). Callose content was expressed as pachyman equivalents (PE) per tissue fresh weight (µg PE·mg_{fw}^{-1}). Data was log transformed to compensate for heterogeneity of variance and analyzed by General Linear Model procedure available on the SAS (version 6.03; SAS 1989). Statistical significance was defined at the 0.05 probability level.

2.2.4. Callose Inhibition and Competitive Inhibition

The following procedures were adapted from Jaffe & Goren (1988). Aluminum-sensitive 'Scout-66' was grown in solution culture, as described above, and treated with the callose inhibitor 2-deoxy-D-glucose (DDG) at 0, 10^{-5}, 5×10^{-5}, or 10^{-4} M at two levels of Al (0 and 400 μM), in triplicate, for a total of 24 experimental units. This monosaccharide inhibits protein glycosylation in influenza-virus infected chick cells, and may act on lipid-linked oligosaccharides (Datema & Schwarz 1979). To further verify that DDG was affecting callose deposition, 'Scout-66' was treated with the DDG competitor mannose at 0 and 5×10^{-5} M at two levels of Al (0 and 400 μM) and at two levels of DDG (0 and 5×10^{-5} M), in triplicate, for a total of 24 experimental units. Mannose acts as a glucose analog and competes with DDG (Datema & Schwarz 1979) to counteract callose inhibition (Jaffe & Goren 1988). Mannose was selected as it was the only DDG inhibitor found in the literature.

2.3. Results

General Plant Morphology and Organ Lengths

Although it was not the purpose of this study to observe morphological changes due to Al toxicity, some observations were made during organ length harvest. Roots of Al-treated plants exhibited typical symptoms of Al toxicity including brown root tips, brittle tissue, and stunted seminal roots. These symptoms were more pronounced in Al-sensitive lines than Al-resistant lines. No symptoms of Al toxicity were observed in the leaves of seedlings.

Root length was reduced in all four wheat lines treated with Al, although this effect was more pronounced in the Al-sensitive lines 'Scout-66' and 'Katepwa' (Figure 2-1). Root length was significantly reduced by Al treatments as low as 30 μM in 'Scout-66' (23% less than control), 100 μM in 'Katepwa' (26%), 700 μM in 'Atlas-66' (30%), and 1000 μM in PT741 (25%). There was a significant interaction effect between Al and

wheat line: the root lengths of the two Al-tolerant lines were less affected by Al treatment than were the two Al-sensitive lines. Maximum reduction of root length occurred at 1000 μM Al with a loss of 60% in 'Katepwa' and 'Scout-66', 40% in 'Atlas-66', and 25% in PT741. A significant linear relationship between root length and Al treatment was detected ($r = -0.60$ to -0.68; $\alpha = 0.05$). A quadratic model was also applied with only slightly better correlation values ($r = -0.60$ to -0.74; $\alpha = 0.05$). A more complete description of the shape of the relationship between root length and Al treatment would require additional experimentation.

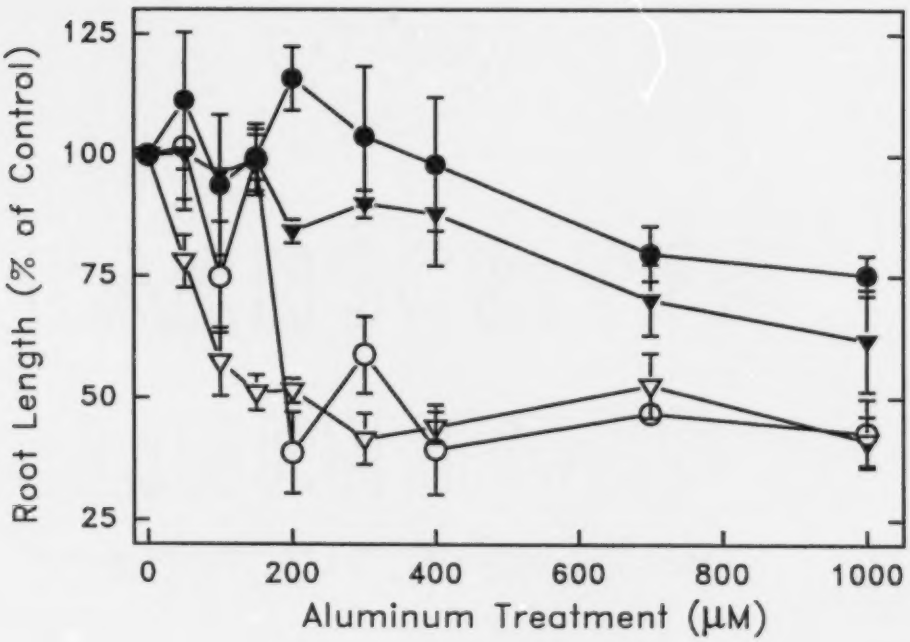

Figure 2-1: Effect of aluminum on root length in four wheat lines: Al-resistant lines 'Atlas-66' (solid triangle), PT741 (solid circle) and Al-sensitive lines 'Scout-66' (hollow triangle), 'Katepwa' (hollow circle). Data are averages of three measurements, pooling of five plants per replicate ± the standard error of the mean. Control values for root length: 'Atlas-66' (21.2 ± 0.8 mm), PT741 (17.9 ± 2.0 mm), 'Scout-66' (21.7 ± 1.0 mm), 'Katepwa' (18.0 ± 1.3 mm).

Leaf length was reduced by treatment with Al in PT741 and 'Scout-66', however this trend was variable (Figure 2-2). No significant interaction effect between wheat line and Al was detected. Leaf length was significantly reduced by Al treatment as low as 30 µM in 'Scout-66' (18%) and 60 µM in PT741 (22%). No trend was discerned in 'Katepwa', and leaf length did not decline in 'Atlas-66' after treatment with up to 1000 µM Al. Despite this, a weak linear relationship was detected between leaf length and Al treatment ($r = -0.31$ to -0.45; $\alpha = 0.05$). A quadratic model gave a slightly better fit ($r = -0.34$ to -0.49; $\alpha = 0.05$). A more complete description of the shape of the relationship between leaf length and Al treatment would require additional experimentation.

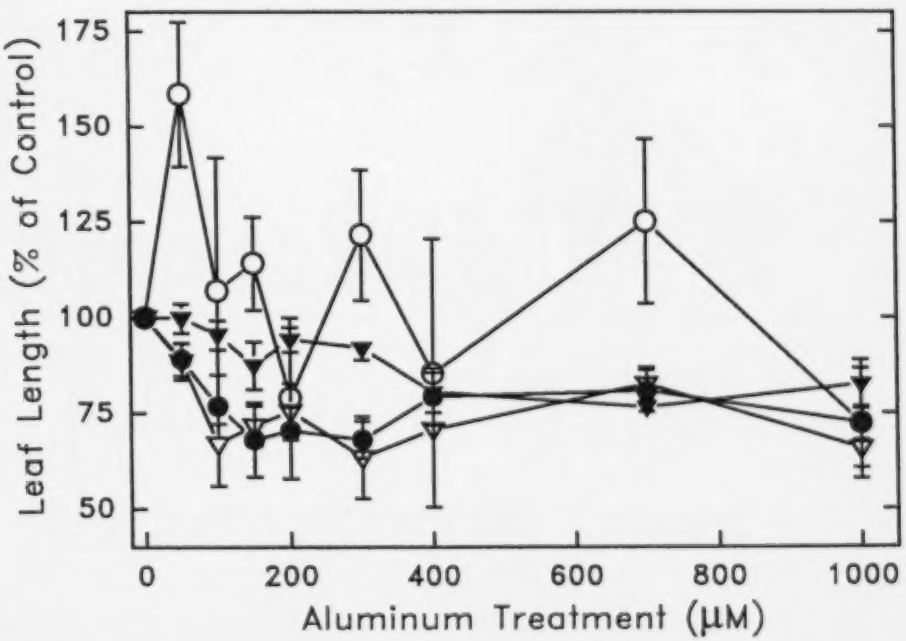

Figure 2-2: Effect of aluminum on leaf length in four wheat lines: Al-resistant lines 'Atlas-66' (solid triangle), PT741 (solid circle) and Al-sensitive lines 'Scout-66' (hollow triangle), 'Katepwa' (hollow circle). Data are averages of three measurements, pooling of five plants per replicate ± the standard error of the mean. Control values for leaf length: 'Atlas-66' (16.6 ± 0.4 mm), PT741 (20.7 ± 0.8 mm), 'Scout-66' (25.8 ± 1.7 mm), 'Katepwa' (7.3 ± 1.5 mm).

Organ callose content and root dose response

Various steps in the spectrofluorometric method of determining callose content were tested for appropriateness to this study. The addition of DDG to the ethanol soak only slightly reduced callose deposition; 'Scout-66' roots grown in 50 μM Al had approximately 6% less callose with DDG in the ethanol than without it in the ethanol. The standard curve was linear up to 4 μg pachyman; full scale fluorescence was not observed up to this concentration. A mixture of root tissue homogenate plus an aliquot of pachyman (PE) standard had a 105% (± 5% standard error) recovery of fluorescence. This indicated that the tissue did not interfere with fluorescence.

The quantification study found that root callose content increased after Al treatment, whereas leaf callose content remained low (Table 2-1). There was a significant interaction effect between Al and organ on organ callose content, however no interaction was detected between Al and wheat line, perhaps due to the small sample sizes. Nevertheless, a more pronounced increase in root callose content was detected in Al-sensitive 'Scout-66' than 'Atlas-66'. Root callose content increased by 140% in 'Atlas-66' and 730% in 'Scout-66' at 400 μM. Leaf callose content was near the minimum level of detection for the spectrofluorometer, despite using a concentrated leaf homogenate containing nearly 70% more tissue by fresh weight than an average root homogenate. Due to the low level of fluorescence in the leaves, subsequent callose content studies involved only roots.

Table 2-1: Effect of aluminum on root and leaf callose content in Al-resistant 'Atlas-66' and Al-sensitive 'Scout-66'.

	CALLOSE CONTENT ($\mu g\ PE \cdot mg_{fw}^{-1}$)			
Al level	'Atlas-66' (Al-resistant)		'Scout-66' (Al-sensitive)	
(μM)	Root	Leaf	Root	Leaf
0	0.16 ± 0.02 c	0.01 ± 0.01 d	0.18 ± 0.00 c	0.01 ± 0.00 de
50	0.15 ± 0.03 c	0.02 ± 0.00 d	0.28 ± 0.01 c	0.01 ± 0.00 d
400	0.38 ± 0.03 b	0.02 ± 0.00 d	1.48 ± 0.01 a	0.01 ± 0.00 de

NOTE: Values are means of two replicates, pooling five to eight plants per replicate, ± the standard error of the mean. Values followed by the same letter are not significantly different at the 5% level, as determined by a Least Squares Means analysis of log transformed data. Abbreviation: (PE) pachyman equivalents.

Root callose content of the four wheat lines increased with Al treatment in a dose-dependent manner, although this was more pronounced in Al-sensitive 'Scout-66' and 'Katepwa' (Figure 2-3). Unlike Table 2-1, a significant interaction effect between Al and wheat line was detected; the root callose content of 'Scout-66' was more sensitive to Al treatment than in any other wheat line. Root callose content was significantly increased by Al treatments as low as 70 μM Al in 'Scout-66' (160% increase), 200 μM Al in 'Katepwa' (340%), 300 μM Al in PT741 (190%), and 400 μM Al in 'Atlas-66' (90%). This ranking of plants by Al sensitivity was similar to that of the root length study, although the order of PT741 and 'Atlas-66' was reversed. On average, nearly three times more Al was required to induce a significant change in root callose content than in root length. However, differences in root callose content between Al-resistant and Al-sensitive lines after Al treatment were larger among winter wheats ('Atlas-66, 'Scout-66') and among spring wheats (PT741, 'Katepwa') than for root length. The maximum

root callose content occurred at 1000 μM Al with an increase of 1100% in 'Katepwa', 900% in 'Scout-66' and PT741, and 240% in 'Atlas-66'. A strong linear relationship was detected between root callose content and Al treatment ($r = 0.82$ to 0.95, $\alpha = 0.05$). A quadratic model was also applied ($r = 0.84$ to 0.88, $\alpha = 0.05$). A more complete description of the shape of the relationship between root callose content and Al treatment would require additional experimentation.

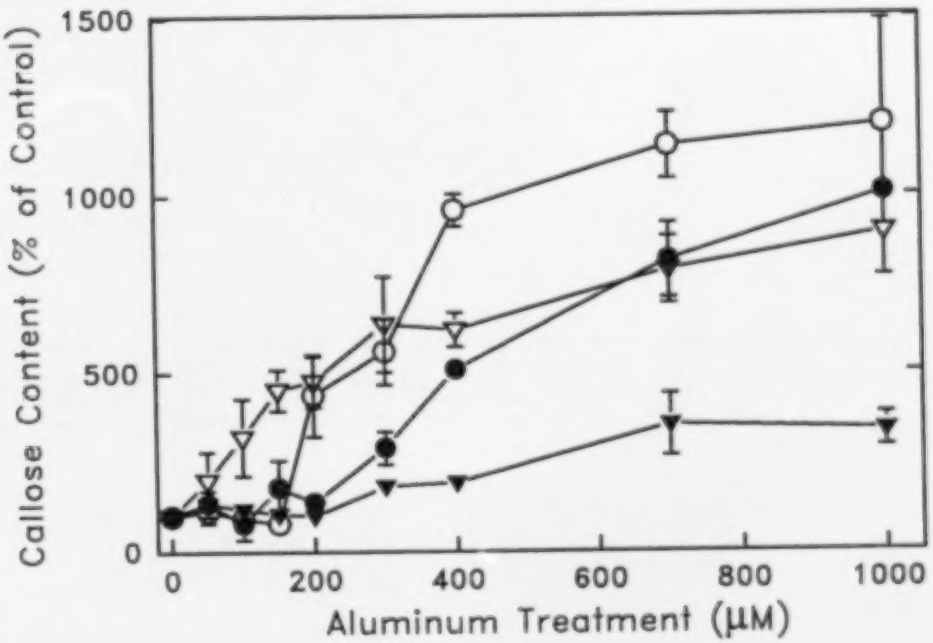

Figure 2-3: Effect of aluminum on root callose content in four wheat lines grown separately: Al-resistant lines 'Atlas-66' (solid triangle), PT741 (solid circle) and Al-sensitive lines 'Scout-66' (hollow triangle), 'Katepwa' (hollow circle). Data are averages of three measurements, pooling of five to eight plants per replicate, ± the standard error of the mean. Control value for callose content: 'Atlas-66' (0.12 ± 0.03 PE), PT741 (0.10 ± 0.01 PE), 'Scout-66' (0.17 ± 0.02 PE), 'Katepwa' (0.17 ± 0.01 PE). Abbreviation: (PE) pachyman equivalents per unit fresh weight (μg PE·mg_{fw}^{-1})

Since harvesting procedures prohibited growing all plants simultaneously, and growth conditions may have varied slightly from day to day, a second experiment was conducted with all four lines, but with fewer Al treatments. This design permitted the direct comparison of Al-resistant and Al-sensitive lines grown under nearly identical conditions. As in Figures 2-1 and 2-3, a significant interaction effect was detected between Al and wheat line on root length and on root callose content; as in Figure 2-2, no interaction effect was detected between Al and wheat line on leaf length (organ length data not presented; Figure 2-4). Root callose content increased in all lines at 400 μM Al, including an increase of 915% in 'Katepwa', 730% in 'Scout-66', 220% in PT741, and 140% in 'Atlas-66'. Higher levels of callose suggested greater membrane injury, according to models of callose deposition (Kauss 1990, Ohana *et al.* 1992), hence this ranking was similar to those of the previous root callose content studies and root length, although the order of the Al-resistant lines was reversed from the root length study.

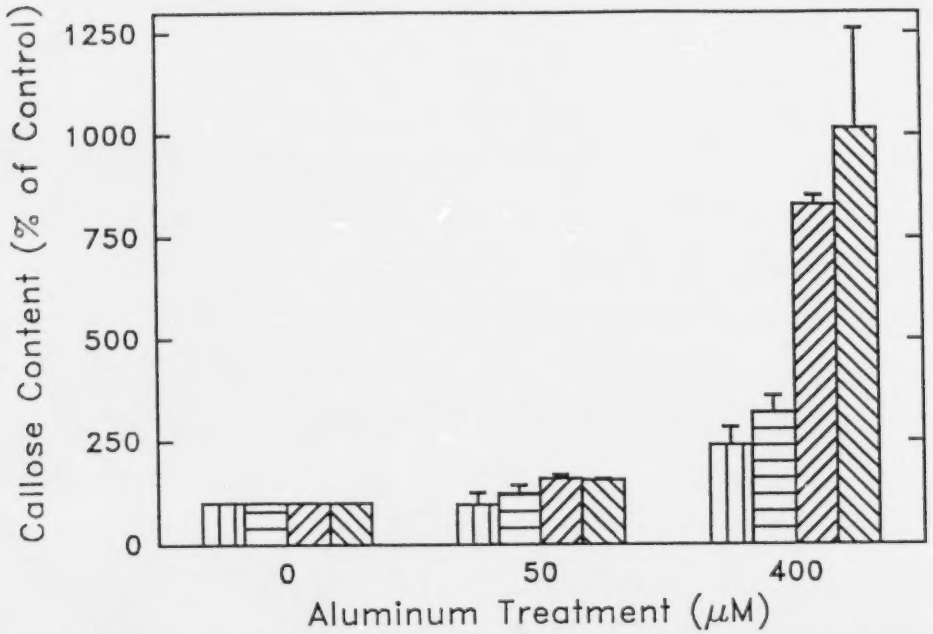

Figure 2-4: Effect of aluminum on root callose content in four wheat lines grown simultaneously: Al-resistant lines 'Atlas-66' (vertical lines), PT741 (horizontal lines) and Al-sensitive lines 'Scout-66' (///), 'Katepwa' (\\\). Data are averages of three measurements, pooling of five to eight plants per replicate, ± the standard error of the mean. Control value for callose content: 'Atlas-66' (0.16 ± 0.02 PE), PT741 (0.17 ± 0.03 PE), 'Scout-66' (0.18 ± 0.00 PE), 'Katepwa' (0.14 ± 0.00 PE). Abbreviation: (PE) pachyman equivalents per unit fresh weight ($\mu g\ PE \cdot mg_{fw}^{-1}$)

Callose Inhibition and Competitive Inhibition

Wheat seedlings were injured by treatment with DDG, despite testing a range of DDG concentrations (10^{-5} M to 10^{-1} M, data not shown) on plant growth in a preliminary study. Plants treated with DDG had dark brown, 'burnt' root tips, stunted, brittle roots, and slightly chlorotic leaves. Increasing concentrations of DDG in the nutrient solution generally caused a corresponding decrease in root length, leaf length, and root callose content in 'Scout-66' (Table 2-2). In the absence of Al, treatment with 10^{-4} M DDG reduced root length by as much as 60%, leaf length by 45%, and callose content by 55%. In the presence of 400 μM Al and 10^{-4} M DDG these percentages were changed to 18%, 35%, and 80%, respectively. No interactions were detected between Al and DDG on root length, nor leaf length nor root callose content.

Table 2-2: Effects of aluminum and callose inhibitor (2-deoxy-D-glucose) on root length, leaf length, and root callose content in Al-sensitive 'Scout-66'.

DDG (M)	Root Length (mm)	Leaf Length (mm)	Root Callose ($\mu g\ PE \cdot mg_{fw}^{-1}$)
0 μM Al			
Control	29.6 ± 1.4 a	42.3 ± 1.4 a	0.16 ± 0.01 c
10^{-5}	27.0 ± 0.5 b	39.5 ± 1.9 a	0.06 ± 0.02 d
$5x10^{-5}$	13.4 ± 0.7 c	25.7 ± 0.3 b	0.07 ± 0.01 d
10^{-4}	11.2 ± 0.4 cd	22.3 ± 0.7 bd	0.07 ± 0.00 d
400 μM Al			
Control	13.1 ± 0.6 ce	29.3 ± 1.8 bc	1.18 ± 0.06 a
10^{-5}	12.7 ± 0.8 c	27.3 ± 1.9 cd	0.64 ± 0.01 b
$5x10^{-5}$	10.8 ± 0.2 ce	23.7 ± 2.7 bd	0.43 ± 0.01 b
10^{-4}	11.4 ± 0.5 de	19.2 ± 1.7 d	0.24 ± 0.07 c

NOTE: Values are means of three measurements, pooling of five to eight plants per replicate, ± the standard error of the mean. Values in a column followed by the same letter are not significantly different at the 5% level as determined by a Least Squares Means analysis of untransformed root and leaf lengths, and log transformed root callose content. Abbreviations: (DDG) 2-deoxy-D-glucose, (PE) pachyman equivalents.

Further confirmation of the presence of callose was tested using mannose, a competitor of DDG, to moderate the effects of DDG in Al-sensitive 'Scout-66'. The effect of adding mannose to the nutrient solution was not significant, nor were any interactions detected between mannose and DDG on root length, nor leaf length, nor root callose content. Nevertheless, mannose did reduce root length and root callose content in the absence of Al (Table 2-3). In contrast, DDG was significant overall and

had a significant interaction effect with Al on root length, leaf length and root callose content. As in previous studies, Al significantly affected all three parameters.

Table 2-3: Effects of aluminum, callose inhibitor (2-deoxy-D-glucose), and DDG competitive inhibitor (mannose) on root length, leaf length and root callose content in Al-sensitive 'Scout-66'.

Treatment	Root Length (mm)	Leaf Length (mm)	Root Callose ($\mu g\ PE \cdot mg_{fw}^{-1}$)
0 μM Al			
Control	28.6 ± 1.8 a	41.2 ± 0.6 a	0.18 ± 0.00 d
Man	25.1 ± 0.7 b	38.1 ± 1.3 a	0.13 ± 0.01 c
DDG	13.8 ± 0.3 d	27.6 ± 1.0 bd	0.08 ± 0.01 e
DDG + Man	16.8 ± 0.9 c	30.9 ± 0.9 bc	0.08 ± 0.01 e
400 μM Al			
Control	13.0 ± 0.7 d	30.4 ± 0.9 bc	1.14 ± 0.12 a
Man	13.0 ± 0.8 d	30.7 ± 1.8 c	1.01 ± 0.13 a
DDG	13.2 ± 0.4 d	24.1 ± 1.5 d	0.41 ± 0.02 b
DDG + Man	12.0 ± 0.7 d	24.3 ± 1.5 d	0.45 ± 0.01 b

NOTE: Values are means of three measurements, pooling of five to eight plants, ± the standard error of the mean. Values in a column followed by the same letter are not significantly different at the 5% level as determined by a Least Squares Means analysis of untransformed root and leaf lengths, and log transformed root callose content. Abbreviations: (Man) 10^{-4} M mannose, (DDG) $5x10^{-5}$ M 2-deoxy-D-glucose, (PE) pachyman equivalents.

2.4. Discussion

The effect of Al on root length for the four lines was similar to those recorded in previous studies (Briggs *et al.* 1989), with 'Katepwa' and 'Scout-66' being more sensitive to lower concentrations of Al than PT741 and 'Atlas-66'. Change in leaf length, however, was a poor indicator of Al toxicity due to its high degree of variability (Figure 2-2). This variability might have been reduced if older plants were used instead of three-day old seedlings since more uniform plants with better vigour could then have been selected. Growth response, such as change in organ lengths, is affected by many factors and the time lag between the onset of stress and a plant's growth response is considerable (Jorns *et al.* 1991). Bennet & Breen (1991) suggest that the accuracy of screening for plant resistance will improve by using a response closer to the sites of stress perception.

Callose deposition is closely associated with membrane injury, and Al is known to damage membranes (Caldwell 1989). Differences in root callose content of the four wheat lines were large and gave a ranking of Al resistance that was similar to published reports (Briggs *et al.* 1989). There was, however, a discrepancy in the ranking of PT741 and 'Atlas-66' which might have been because Briggs *et al.* (1989) used a single concentration of Al, 75 μM, to rank plant responses. It was possible that PT741 and 'Atlas-66' had different optimum Al concentrations that affected their relative ranking. Alternatively, the three-day old seedlings used in this study might have responded differently to Al than the fourteen day old plants used by Briggs *et al.* (1989).

Both root length and root callose content were useful in ranking plants for Al resistance. Root length was more sensitive to low levels of Al, whereas differences between plants were larger with root callose content. For example, compared to Al-free controls, root length in the two Al-sensitive lines first significantly decreased after

treatment with 50 μM Al by an average of 23%; root callose content first significantly increased after treatment with 150 μM Al by an average of 250%. Root callose content was also associated with a larger variance than for root length, but this would likely be reduced after refinements to the harvesting protocol.

The harvesting protocol likely led to an overestimate of root callose content. Although tissues were handled carefully, some callose was deposited before tissue fixation, as seen in other studies (Galway & McCully 1987; Chapter 3). Callose was also likely deposited during the ethanol presoak. Alcohol is a good fixative but it takes time to penetrate the tissue (Jensen 1962). At least 6% more callose was detected when the roots of 'Scout-66' were presoaked in ethanol alone, than when callose inhibitor, 2-deoxy-D-glucose (DDG), was added to the ethanol. Despite this, the trend of increased callose deposition with Al treatment was likely unaffected.

In contrast to the root callose content, leaf callose content did not significantly change with Al, and was near the limit of detection of the spectrofluorometer. Oat mesophyll cell protoplasts, however, synthesize callose after Al treatment (Schaeffer & Walton 1990) and callose was observed in 'Scout-66' squashed leaves (Chapter 3 Figure 3-18), thus leaf cells are capable of synthesizing callose. The lack of callose deposition in leaves suggested that little Al-induced injury occurred at the leaf membrane and that toxic species of Al were not reaching these plant parts. Nevertheless, Al does induce some leaf injuries, including reduced photosynthesis on a leaf area basis (Scott *et al.* 1991).

This study did not provide evidence of a positive correlation between callose deposition and Al resistance. This was consistent with reports that mesophyll cell protoplasts of Al-sensitive wheat line 'Scout-66' have more callose, as estimated by [^{14}C] glucose incorporation, than do those of Al-resistant 'Atlas-66' after Al treatment (Schaeffer & Walton 1990). Due to the rapidity of callose deposition and degradation (Aist 1977), it was possible that early deposits occurred in the Al-resistant line and

were already hydrolysed by the plant before being harvested. In such a manner, callose might have provided a temporary, transient form of resistance until another resistance mechanism took over. A time course study would address the possibility of early callose deposition in Al resistant lines.

The distribution of Al in Al-sensitive and Al-resistant plants of (Rincon & Gonzales 1992) fitted the observations of differential callose deposition of this study. Using hematoxylin staining and atomic absorption spectrophotometry to detect Al, Rincon & Gonzales (1992) found that Al-resistant 'Atlas-66' had much less Al in its root meristems than Al-sensitive 'Tam 105'. With less Al present to induce membrane damage, less callose would have been deposited in the roots of Al-resistant lines, as observed in this study. Perhaps the Al-resistant plants prevented toxic species of Al from entering the root. Previous studies found that the plasma membrane is an important barrier to the passive movement of Al (Wagatsuma 1983). Alternatively, it was possible that the Al-resistant plants were able to prevent Al-induced damage to the plasma membrane.

Interestingly, some evidence suggests that callose could not have been deposited since the required calcium influx may not occur after Al treatment. Calcium may be blocked from entering the symplasm of the root apex of 3-day old Al sensitive 'Scout-66' (Huang *et al.* 1992). These results were recently questioned by Ryan *et al.* (1992) since they found that Al generally has no effect on calcium influx and that because the calcium measurements approached the detection limit of the system, no conclusions can be drawn from these results. Nevertheless, if the study by Huang *et al.* (1992) gained further experimental support, the results of this study may still be reconciled. It is possible that calcium might be undetected by the microelectrodes if this cation was stored in organelles (Kauss 1990) or if the ion flux varied along the length of the root. Root callose content was determined from whole roots. And finally, it was possible that the compound measured in this study was not callose, but another

polysaccharide with similar fluorescing properties. This, however, seemed unlikely given the results of the DDG study and the callose localization study in Chapter 3.

Root callose content was reduced after treatment with DDG in a dose-dependent manner (Table 2-2). Although the action of this compound is not well-known, DDG is likely metabolized into UDP-deoxyglucose and GDP-deoxyglucose (Datema & Schwarz 1979). In viruses and algae, these metabolized forms inhibit first biosynthesis of lipid-linked oligosaccharides, then protein glycosylation - the transfer of completed lipid-oligosaccharides to the protein (Datema & Schwarz 1979, Datema *et al.* 1983). This compound also inhibits gravitropism and tendril coiling in *Pisum sativum*, and callose deposition in *P. sativum* (Jaffe & Leopold 1984) and *Phaseolus* stems (Jaffe *et al.* 1985).

Such a general action might explain the observed toxic responses of DDG on wheat seedlings, including reduced organ lengths and root damage. However, no toxic effects were reported by Jaffe & Leopold (1984) after 10 µM DDG was applied to the roots of *Pisum sativum* and *Zea mays*; and, up to 10^{-2} M DDG was not "excessively toxic" to leaf abscission in *Citrus sinensis* (Jaffe & Goren 1988). Fewer toxic effects might have been observed if wheat seedlings were treated with DDG for shorter periods of time. Without additional knowledge of the effects of DDG on wheat it was difficult to speculate how the observed toxic responses impacted on these results.

An additional study to confirm callose presence using mannose, the DDG competitive inhibitor, was inconclusive. Lipid-linked mannose participates in protein glycosylation and mannan formation in *Prototheca zopfii* and exogenous mannose is reported to compete with DDG for an active site in protein glycosylation (Datema & Schwarz 1979, Datema *et al.* 1983). Consequently, mannose was expected to reduce the inhibitory effects of DDG on callose deposition. This expectation was not observed, although a higher concentration of mannose might have been required for such a response. Alternatively, since mannose decreases the movement of phosphates in

some C3 plants (Herold *et al.* 1976), it might have induced a phosphate deficiency in the plants of this study and lower glucose availability in lower plant parts. Without glucose, callose could not be synthesized. Further study was needed to confirm the presence of callose in wheat roots. This was addressed in Chapter 3.

In summary, callose was not positively correlated with Al resistance. Leaf length was a poor indicator of Al resistance, and although root length was more sensitive to lower Al levels, larger differences were observed in root callose content between the Al-resistant and Al-sensitive lines. These results suggested that Al resistance might occur at the root level. Root callose deposition might be useful in short-term physiological studies of membrane injury or in breeding programs as a sensitive screen for Al resistance. Because callose deposition is a response to membrane injury, it might also be used as a biological indicator of the passage of toxic compounds like Al, as described in Chapter 3. These results supported previous studies suggesting that root responses were more sensitive to Al than leaf responses (Taylor & Foy 1985, Briggs *et al.* 1987).

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3. Localization of Callose in Aluminum-Treated Wheat[1]

3.1. Introduction

Traditionally, callose deposition has been of most interest to plant pathologists and phloem researchers (Currier 1957), because it occurs at pathogen infection sites, sieve plates, and plasmodesmata (Aist 1977). Callose is a β-1,3 glucose polymer which is induced after plasma membrane disturbances cause a localized influx of calcium ions (Kauss 1990) and release vacuolar forms of β-furfuryl-β-glucoside (Ohana *et al.* 1992). This cation and β-glucoside bind to separate sites on the cytoplasmic side of the membrane-bound callose synthase (Ohana *et al.* 1992, Fredrikson & Larsson 1989), and induce the extracellular deposition of callose. Since callose deposition occurs after injury at the plant membrane, it may be used in stress physiology studies as a marker for the transport of harmful substances (Currier 1957). For example, the pattern of callose deposition in *Phaseolus vulgaris* provided clues to the movement of boron along the transpiration stream (McNairn & Currier 1965).

This study examined the location of callose deposits in wheat (*Triticum aestivum* L.) to provide clues as to the movement of toxic species of aluminum (Al). Chapter 2 found that after Al treatment, root callose content may be used to distinguish between Al-resistant and Al-sensitive wheat plants. That study, however, did not indicate where the callose was being deposited nor how far toxic Al species penetrated the plant. The present study addressed these two questions using fluorescence microscopy on the freeze-substituted and squashed roots and leaves of wheat.

Studying callose deposition by fluorescence microscopy is difficult. Not only is callose deposition induced by handling and conventional tissue fixation, its

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[1] A version of this chapter is being prepared for publication.

identification by staining has been questioned (Smith & McCully 1978). These concerns were addressed in this study by comparing the distribution of callose in freeze-substituted tissues with that in chemically-fixed tissues, using Sirofluor as a relatively callose-specific stain. Further, callose presence was confirmed by adding callose inhibitor to the nutrient solutions of plants, and callose hydrolase to embedded tissues. As in the quantitative study in Chapter 2, there was no evidence to support a positive correlation between callose deposition and Al resistance. Little callose was deposited in the root tips and leaves of Al resistant lines. This suggested that toxic species of Al were prevented from reaching these tissues in the resistant line, and that a resistance mechanism may be operating at the periphery of the roots.

3.2. Materials and Methods

Seeds of two Al-resistant lines ('Atlas-66' & PT741) and two Al-sensitive lines ('Scout-66' & 'Katepwa') of *Triticum aestivum* L. were germinated and grown in solution culture, as previously described (Chapter 2.2. 'Methods and Materials'). Plants were treated for 40 hours with 0 or 400 μM Al, in triplicate, for a total of 24 experimental units. An additional 12 experimental units included only the lines 'Scout-66' and 'Atlas-66' treated with the callose inhibitor 2-deoxy-D-glucose (DDG, 5×10^{-5} M) at 0 or 400 μM Al, in triplicate. The grand total of 36 experimental units were grown in a randomized block design, with overlapping growth conditions. For each beaker, five plants were fixed by freeze-substitution and prepared for embedding and fluorescence microscopy. Five different plants were used to determine the general plant morphology, mean maximum root length, and mean leaf length. Maximum root length was determined for the longest seminal root; leaf length was determined from seed emergence to the tip of the oldest blade. Untransformed data were analyzed by the General Linear Model and Analysis of Variance procedures available on the Statistical

Analysis System (SAS version 6.03; SAS 1989). Statistical significance was defined at the 0.05 probability level.

A second experiment was conducted to compare the patterns of callose deposition in tissues fixed chemically and by freeze-substitution. Aluminum-sensitive 'Scout-66' and Al-resistant 'Atlas-66' were treated with 0 or 5 x 10^{-5} M 2-deoxy-D-glucose (DDG) at 0 and 400 μM Al, without replication, for a total of eight experimental units. For each beaker, five plants were chemically fixed and squashed for fluorescence microscopy; as before, five different plants were harvested for organ lengths.

3.2.1. Freeze-substitution, Embedding, and Sectioning

Excised tissues from the distal 5 mm root tip, (contained in porous coffee filter pouches to prevent tissue loss), and the mid 10 mm leaf segment were plunged into a semi-viscous mixture of 12% methylcyclohexane in 2-methyl butane cooled by liquid nitrogen. At this temperature (approximately -175^{o}C), sub-microscopic ice crystals were formed from cellular water, and later withdrawn by dissolving in an anhydrous solution of 1.3% acrolein over molecular sieves (type 13X, Sigma Chemical Co., St Louis, Missouri, USA), in acetone cooled by dry ice for 13 days (Hughes & Gunning 1980, Humbel & Muller 1985). Acrolein rapidly prevents callose deposition by cross-linking proteins (Feder & O'Brien 1968, Galway & McCully 1987). Tissues were then brought slowly to room temperature for embedding.

Freeze-substituted tissues were embedded in glycol methacrylate (GMA) according to Galway & McCully (1987) using a modified GMA mixture (Spaur & Moriarty 1977, Table II; Brander & Wattendorff 1989). Plant material was slowly infiltrated in an acetone-GMA series and soaked overnight in unpolymerized GMA. Tissues were transferred to size 00 gelatin capsules containing partly-polymerized

GMA, vacuum-infiltrated for 10 minutes, capped, and polymerized by irradiation from a long wavelength (>315 nm) ultraviolet light source (Osram Ultra Vitalux lamp 300 W, 10 cm away) for 15 - 20 hours at 10°C in the dark. Covering the capsules prevented oxygen from inhibiting GMA polymerization (Feder & O'Brien 1968). The blocks were then sectioned at 1 µm using glass knives on a Reichert-Jung Ultracut microtome in a 10% acetone-water bath and mounted on gelatin-coated slides (Jensen 1962) for staining and fluorescence microscopy.

3.2.2. Chemical Fixation, Clearing, and Squashing

Excised tissues from the distal 5 mm root tip and mid 10 mm leaf segment were chemically fixed according to Jaffe & Leopold (1984) in a 1:1:8 (v:v:v) mixture of 40% formalin, acetic acid, and 95% ethanol for two days under vacuum infiltration. Tissues were then softened by heating for 5 hours at 60°C, rinsed in distilled water, cleared with 8 M NaOH for 60 minutes at 60°C, and stored in 0.067 M potassium phosphate buffer (pH 8.5) prior to staining and fluorescence microscopy. Jaffe and Leopold (1984) found that tissues may remain in this condition for several months without further callose deposition.

3.2.3. Staining and Fluorescence Microscopy

Before staining, sectioned tissues were soaked in a saturated solution of 2,4-dinitrophenylhydrazine (DNPH, Calbiochem,USA) in 15% (v/v) acetic acid in distilled water for 10 minutes, then rinsed in distilled water for 10 minutes. This procedure blocked the Schiff-positive aldehyde groups introduced by acrolein (Feder & O'Brien 1968) and reduced the binding of the dye to the plastic (Smith & McCully 1978). Afterwards, both sectioned and cleared tissues were pre-stained with 1% (w/v) periodic acid for 10 minutes, rinsed in distilled water for 5 minutes, followed by Schiff's reagent

(Fisher Scientific, USA) for 20 minutes, 3 baths of 0.5% sodium metabisulfite of 2 minutes each, and a final rinse in distilled water for 5 minutes (Feder & O'Brien 1968).

Callose was stained for 30 minutes with either 0.1% (w/v) aniline blue (water soluble, C.I. #42755, lot # 60894; PolySciences, Pa, USA) in cleared tissues, or with 0.003% (w/v) Sirofluor ([sodium 4,4'[carbonylbis(benzene-4,1-diyl)bis(imino)]bisbenzene sulfonate; Biosupplies, Parkville, Victoria, Australia) in 0.067 M potassium phosphate buffer (pH 8.5) in sectioned tissues. Tissues were viewed by epifluorescence using a Zeiss fluorescence photomicroscope (filter pak II consisting of a G365 excitation filter) for UV fluorescence, equipped with a mercury lamp. No barrier filters were used due to the low fluorescence observed. Fluorescence observations were recorded on 35 mm Kodak TMax 400 (45 second exposure) for squashes, or TMax 3200 (800 ASA, 30 second exposure, unless otherwise specified) for GMA embedded tissues. Phase contrast observations of GMA embedded tissues were recorded on TMax 100 film.

3.2.4. Callose Hydrolase, Cellulose Staining, and Controls

Callose presence was tested by observing fluorescence after enzymic digestion of sections. Prior to PAS staining, representative samples of sectioned tissues were incubated with either 14 unit·mg^{-1} of the callose degrading enzyme β-D-glucoside glucohydrolase ([EC 3.2.1.21]; Sigma Chemical Co., USA) in acetate buffer (20 mM, pH 4.8-5.0) or with buffer alone for 5 or 10 hours at 35°C (Brander & Wattendorff 1989), and then rinsed in distilled water. Sections were then stained for callose with Sirofluor (as described above) or for general cell wall constituents (primarily cellulose) with 0.1% Calcofluor White M2R fluorescent brightner 28 ([C.I. # 40622, lot # 34F0647]; Sigma Chemical Co., USA) for 2 minutes.

3.3. Results

Organ lengths and general observations of embedded and squashed tissues

Plant organ length was reduced by treatment with Al. This trend was more pronounced in the roots than in the leaves, especially for the Al-sensitive lines. There was a significant interaction effect between organ and wheat line, but not organ and Al. After 400 μM Al treatment, root length decreased by 65% in 'Katepwa', 50% in 'Scout-66', 35% in PT741, and 30% in 'Atlas-66'. The leaf length decreased by 40% in 'Katepwa', 35% in 'Scout-66', 20% in PT741, and 15% in 'Atlas-66'. Roots of Al-treated plants exhibited typical symptoms of Al toxicity including brown root tips, brittle tissue, and stunted seminal roots. These symptoms were more pronounced in Al-sensitive lines than Al-resistant lines. No symptoms of Al toxicity were observed in the general leaf morphology of the seedlings.

Some notes were taken regarding the fixation-embedding procedure of plant tissue. Of the embedding media used in preliminary studies, (Epon, paraffin, and glycol methacrylate [GMA]), GMA was most appropriate for examining callose deposition in wheat tissues. Glycol methacrylate made the tissues easily visible, which facilitated the trimming and sectioning of block faces, and GMA was readily penetrated by the water-based aniline blue dye. On the other hand, GMA is a soft medium and tended to tear during the handling of semi-thin sections (arrows, Figures 3-1 and 3-4), whereas thicker sections (2-3 μM) did not tear as readily but had poorer resolution (not shown).

There was little evidence of tissue damage after freeze-substitution in embedded tissues. Cell walls were intact and cells maintained their general alignment, as shown in a typical section of leaf (Figure 3-1) and root (Figures 3-4 and 3-7). However, some damage was observed in chemically-fixed, squashed tissues. For example, 'Scout-66' at 400 μM Al had shrunken root hairs (R, Figure 3-25) and leaf stomata (S, Figure 3-

18), probably due to the alcohol dehydration. Observations of cellular damage due to Al and DDG treatments are discussed in 'Effects of callose inhibitor and hydrolase'.

Callose localization in embedded tissues

'Callose' was defined as that compound which fluoresced green-blue under ultraviolet excitation light (approximately 365 nm) following staining with Sirofluor. However, because callose should fluoresce yellow under these conditions (Stone 1984, Smith & McCully 1978), tissues were also observed under a second fluorescence microscope. A fresh, longitudinal section of *Cucurbita* sp. stem was stained with Sirofluor for several minutes and observed under both microscopes. This plant was a useful independent check on the presence of callose since *Cucurbita* is noted for its conspicuous deposits of callose along the sieve plates. Although a similar pattern of fluorescence was observed between the two microscopes, deposits fluoresced with a yellow-green hue under the second microscope, and green-blue under the microscope used in this study. Therefore callose was detected as blue-green fluorescence. Colour discrepancies appeared to be due to the age and alignment of the mercury lamp, and did not affect the appearance of the pictures on black and white film.

Autofluorescence (fluorescence in the absence of fluorochrome staining) of the cell walls and medium was low in embedded tissues, and was therefore not noticeably affected by prestaining with periodic acid-Schiff reagent (PAS; Figure 3-3). Pre-staining with PAS reduces autofluorescence by staining cellulose and pectins while not affecting the staining of callose with aniline blue (Galway & McCully 1987, Smith & McCully 1978). Occasionally some fluorescence was observed that was associated not with callose in the cells, but with the debris of the embedding media (arrows, Figures 3-7 and 3-8).

Generally, Al treatment was associated with reduced organ lengths and increased organ callose, although leaves were an exception. Leaf length was reduced

after Al treatment; no callose was observed in the leaves (Figure 3-2). Little callose was observed in the roots of either Al-free controls, 'Atlas-66' (Figure 3-5) and 'Scout-66' (Figure 3-8), or Al-treated 'Atlas-66' (Figure 3-6). However, after Al treatment, deposits were observed in the roots of Al-sensitive 'Scout-66' (Figures 3-9, 3-10, and 3-14 to 3-17). Callose was associated with the cells of the root cap (R) and outer cortex (C; Figure 3-9), and was especially dense around the pit fields (arrows, Figure 3-15). A non-median section of a 'Scout-66' root had a similar distribution of callose, although there was also an apparent 'gap' (G) behind the root cap (R), followed by an inner band of callose about four cells wide (Figure 3-14).

Callose localization in tissue squashes

Initially, half of the freeze-substituted tissues were reserved for GMA embedding and the other half for clearing and squashing. However, the acetone dehydration procedure rendered tissues too brittle for squashing; even prolonged treatment with 8 M NaOH did not noticeably soften the tissues, and risked dissolving some of the callose (Clark & Villemez 1972). Consequently, new tissues were fixed chemically in order to compare callose deposition of tissues fixed by two independent methods.

In contrast to the embedded tissues, the autofluorescence of squashed tissues was relatively high (Figure 3-19) and was reduced after PAS staining (Figure 3-20). The greater autofluorescence in squashed tissues over embedded tissues likely reflected an increase in the thickness of tissues being observed. This may also explain the higher intensity of fluorescence observed in the squashed tissues. Leaves, after Al treatment and handling, had the occasional patch of callose (pointer), especially near the leaf hairs (L; Figure 3-18). More callose was observed in the roots of both 'Atlas-66' (Figure 3-22) and 'Scout-66' (Figure 3-24) than the Al-free controls (Figures 3-21 and 3-23, respectively), although this response was more pronounced in the Al-sensitive line

'Scout-66'. As with the embedded tissues, callose was located mainly at the root cap (Figure 3-24), but also along the root hairs (R; Figure 3-25).

Effects of callose inhibitor and hydrolase

Plants treated with DDG had dark brown, 'burnt' root tips, stunted, brittle roots, and slightly chlorotic leaves. Organ lengths were generally reduced in both 'Atlas-66' and 'Scout-66' after treatment with DDG, with the exception of root length of 'Scout-66' at 400 μM Al (Table 3-1). There were significant interaction effects between organ and wheat line, and DDG and Al. For example, in DDG-treated 'Atlas-66', root length was reduced by 70% and 40% in the absence and presence of Al, respectively; in DDG-treated 'Scout-66', root length was reduced by 55% in the absence of Al, and not significantly affected in the presence of Al. There were no significant interaction effects on organ length between wheat line and DDG, nor between DDG and organ, nor between Al and organ.

Callose was also observed in prepared tissues after treatment with DDG. Although callose was observed in the embedded roots of the Al-sensitive line 'Scout-66' after Al treatment (Figures 3-9 and 3-10), little was observed after Al plus DDG treatment (Figure 3-13), and none was observed after DDG treatment alone (not shown). Contradictory results, however, were observed in the squashed tissues. After DDG treatment, squashed roots had more deposits of callose (Figure 3-27) than the DDG-free control (Figure 3-21). At higher magnification this callose appeared to be deposited around the entire cell wall (Figure 3-26). This increase in callose deposits after DDG treatment was more pronounced in the presence of Al. 'Atlas-66' at 400 μM Al plus DDG (Figure 3-28) fluoresced solidly in contrast to the Al-treated but DDG-free control (Figure 3-22).

The presence of callose was also tested, in the embedded roots of Al-treated 'Scout-66', by reduced fluorescence after incubation with callose hydrolase. Less

callose was observed after 10 hours with the enzyme (Figure 3-12) than a similar period with only a buffer (Figure 3-11). General fluorescence of other wall components including cellulose was not noticeably affected (not shown) as determined by staining with calcofluor.

Table 3-1: Effects of aluminum and callose inhibitor (2-deoxy-D-glucose) on root length and leaf length in Al-resistant 'Atlas-66' and Al-sensitive 'Scout-66'.

	ORGAN LENGTH (mm)			
Treatment	'Atlas-66' (Al-resistant)		'Scout-66' (Al-sensitive)	
(μM Al ± DDG)	Root	Leaf	Root	Leaf
0	39.0 ± 2.1 a	50.9 ± 3.3 a	32.3 ± 3.0 a	59.8 ± 3.5 a
0 + DDG	11.9 ± 1.8 c	36.2 ± 4.6 b	13.6 ± 1.6 b	42.7 ± 5.0 b
400	27.1 ± 2.4 b	43.7 ± 6.0 a	14.0 ± 2.4 b	41.4 ± 1.6 b
400 + DDG	15.7 ± 2.2 c	37.0 ± 1.9 b	10.4 ± 0.8 b	30.1 ± 1.1 c

NOTE: Values are the means of three measurements, pooling of five plants, ± the standard error of the mean. Values in a column followed by the same letter are not significantly different at the 5% level as determined by a Least Squares Means analysis. Abbreviation: (DDG) $5x10^{-5}$ M 2-deoxy-D-glucose.

Figures 3-1 to 3-10: Epifluorescence and phase contrast micrographs of 1-µm longitudinal sections of seminal roots tips and mid-leaves from 3-day old *Triticum aestivum* L. The aluminum (Al)-sensitive 'Scout-66' line and Al-resistant line 'Atlas-66' were grown in solution culture with 0 µM or 400 µM Al for forty hours. Tissues were freeze-substituted in acetone, embedded in glycol methacrylate, pre-stained with periodic acid-Schiff reagent (PAS), with or without Sirofluor staining, and photographed with a 30 second exposure time (unless otherwise noted).

Figure 3-1: Phase contrast micrograph of longitudinal section of 'Scout-66' leaf at 400 µM Al (automatic exposure); 120x. Note the intact mesophyll cells, and small tears in the section (arrow).

Figure 3-2: Fluorescence micrograph of longitudinal section of 'Scout-66' leaf at 400 µM Al; 120x. Note the low fluorescence typical of all leaf sections grown in the presence or absence of Al.

Figure 3-3: Autofluorescence micrograph of longitudinal section of 'Scout-66' root at 400 µM Al. Sections were stained with PAS but without Sirofluor, (expose 60 sec); 120x. Note the lack of fluorescence in tissue and embedding medium, even after long exposure time.

Figure 3-4: Phase contrast micrograph of longitudinal section of 'Atlas-66' root at 400 µM Al, (automatic exposure); 120x. Note the starch granules (small arrows) in root cap (R), regular cell alignment, and tears in the section (large arrow).

Figure 3-5: Fluorescence micrograph of longitudinal section of 'Atlas-66' root at 0 µM Al; 120x. Note the low fluorescence.

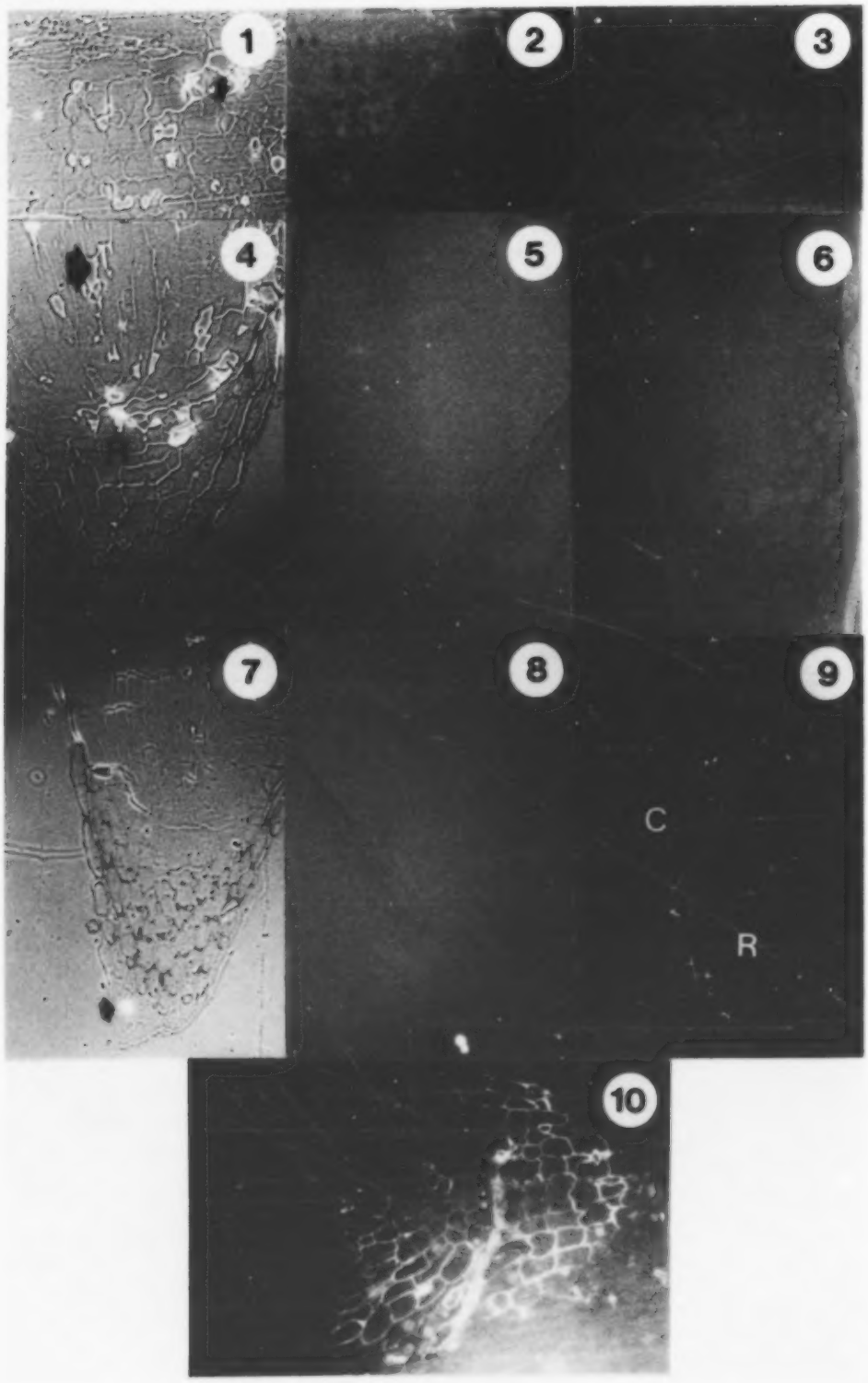
Figure 3-6: Fluorescence micrograph of longitudinal section of 'Atlas-66' root at 400 µM Al; 120x. Note the low fluorescence.

Figure 3-7: Phase contrast micrograph of longitudinal section of 'Scout-66' at 0 µM Al, (automatic exposure); 120x. Note the regular cell alignment and intact cell walls; media debris (arrow) occasionally affected fluorescence as in Figure 3-8.

Figure 3-8: Fluorescence micrograph of longitudinal section of 'Scout-66' at 0 µM Al; 120x. Note the low fluorescence. Spot fluorescence (arrow) was caused by media debris as in Figure 3-7.

Figure 3-9: Fluorescence micrograph of longitudinal section of 'Scout-66' root at 400 µM Al - medium magnification, 120x. Note the strong fluorescence at the root periphery, including root cap (R) and outer cortex (C).

Figure 3-10: Fluorescence micrograph of longitudinal section of 'Scout-66' root at 400 µM Al - high magnification, 175x. Note the strong fluorescence surrounding cell walls.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Figures 3-11 to 3-17: Epifluorescence micrographs of 1-µm longitudinal sections of seminal roots tips from 3-day old *Triticum aestivum* L. The aluminum (Al)-sensitive line 'Scout-66' was grown in solution culture with 400 µM Al for forty hours, with or without the callose inhibitor, $5x10^{-5}$ M 2-deoxy-D-glucose (DDG). Tissues were freeze-substituted in acetone, embedded in glycol methacrylate, incubated with either callose hydrolase or buffer for 10 hours at 35°C, and then stained with periodic acid-Schiff reagent and Sirofluor before being photographed with a 30 second exposure time (unless otherwise noted).

Figure 3-11: Fluorescence micrograph of longitudinal section of 'Scout-66' root at 400 µM Al incubated with acetate buffer; 125x. Note the high fluorescence (pointer) as compared to Figures 3-12 and 3-13.

Figure 3-12: Fluorescence micrograph of longitudinal section of 'Scout-66' root at 400 µM Al incubated with callose hydrolase; 125x. Note the low fluorescence (pointer) as compared to Figures 3-11 and 3-13.

Figure 3-13: Fluorescence micrograph of longitudinal section of 'Scout-66' root at 400 µM Al plus DDG; 125x. Note the low fluorescence as compared to Figure 3-11.

Figure 3-14: Fluorescence micrograph of non-median longitudinal section of 'Scout-66' root at 400 µM Al, 125x. Note the apparent reduced fluorescence or 'gap' (G) between the root cap (R) and the cortex (C).

Figure 3-15: Fluorescence micrograph of longitudinal section of 'Scout-66' root cortical cells at 400 µM Al; 790x. Note the heavy callose deposits surrounding the cortical cells (C), especially at the pit fields (arrows).

Figure 3-16: Fluorescence micrograph of longitudinal section of 'Scout-66' root sieve cells at 400 µM Al, (20 seconds); 790x. Note the heavy callose deposits at what appear to be sieve cells (arrow).

Figure 3-17: Fluorescence micrograph of longitudinal section of 'Scout-66' root cortical cells at 400 µM Al; 790x.

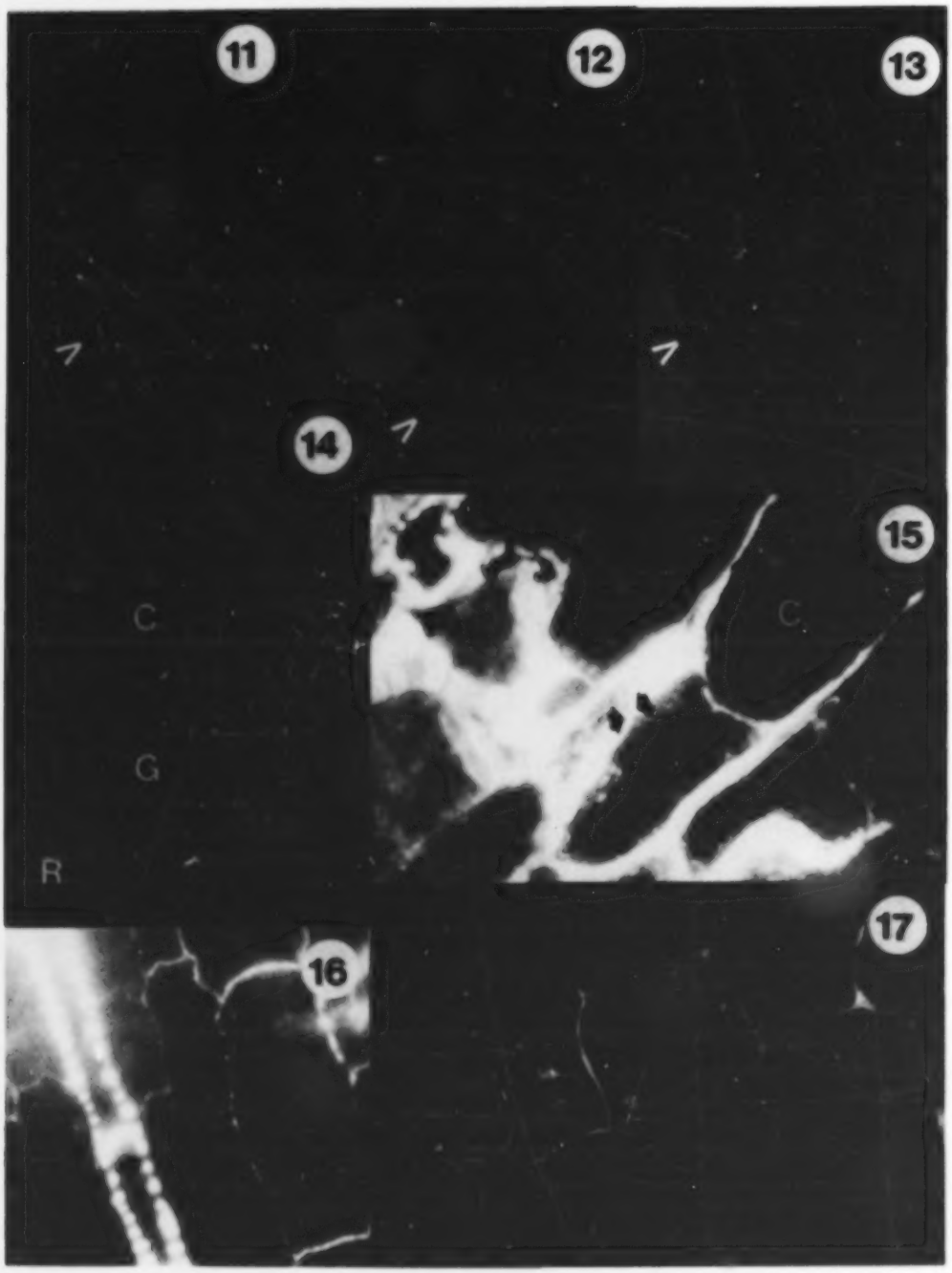
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Figure 3-18 to 3-28: Epifluorescence micrographs of tissue squashes of seminal roots and leaves of two lines of *Triticum aestivum* L. The aluminum (Al)-sensitive line 'Scout-66' and Al-resistant line 'Atlas-66') were grown in solution culture with 0 μM and 400 μM Al, and in the presence or absence of callose inhibitor, $5x10^{-5}$ M 2-deoxy-D-glucose (DDG). Tissues were fixed in 1:1:8 (v:v:v) 40% formalin: acetic acid: 95% ethanol, cleared in 8 M NaOH, stained with periodic acid-Schiff reagent (PAS) and aniline blue. Photographs were exposed for 30 seconds (unless otherwise noted).

Figure 3-18: Fluorescence micrograph of 'Scout-66' leaf squash at 400 μM Al, (45 seconds); 110x. Note the low fluorescence typical of all leaf squashes with or without Al. Small deposits of callose occur in epidermis (pointer), and along leaf hairs (L); stomata appear shrunken (S).

Figure 3-19: Autofluorescence micrograph of 'Scout-66' root squash at 400 μM Al, without staining, (60 seconds); 110x. Note the high autofluorescence as compared to the PAS stained tissue in Figure 3-20.

Figure 3-20: Autofluorescence micrograph of 'Scout-66' root squash at 400 μM Al, with PAS but without aniline blue staining, (60 seconds); 110x. Note the low autofluorescence as compared to the unstained tissue in Figure 3-19.

Figure 3-21: Fluorescence micrograph of 'Atlas-66' root squash at 0 μM Al; 110x. Note the low fluorescence as compared to Figure 3-22.

Figure 3-22: Fluorescence micrograph of 'Atlas-66' root squash at 400 μM Al; 170x. Note the high fluorescence as compared to Figure 3-21.

Figure 3-23: Fluorescence micrograph of 'Scout-66' root squash at 0 μM Al. Negative was made from an ektachrome slide (25 seconds); 170x. Note the low fluorescence as compared to Figure 3-24.

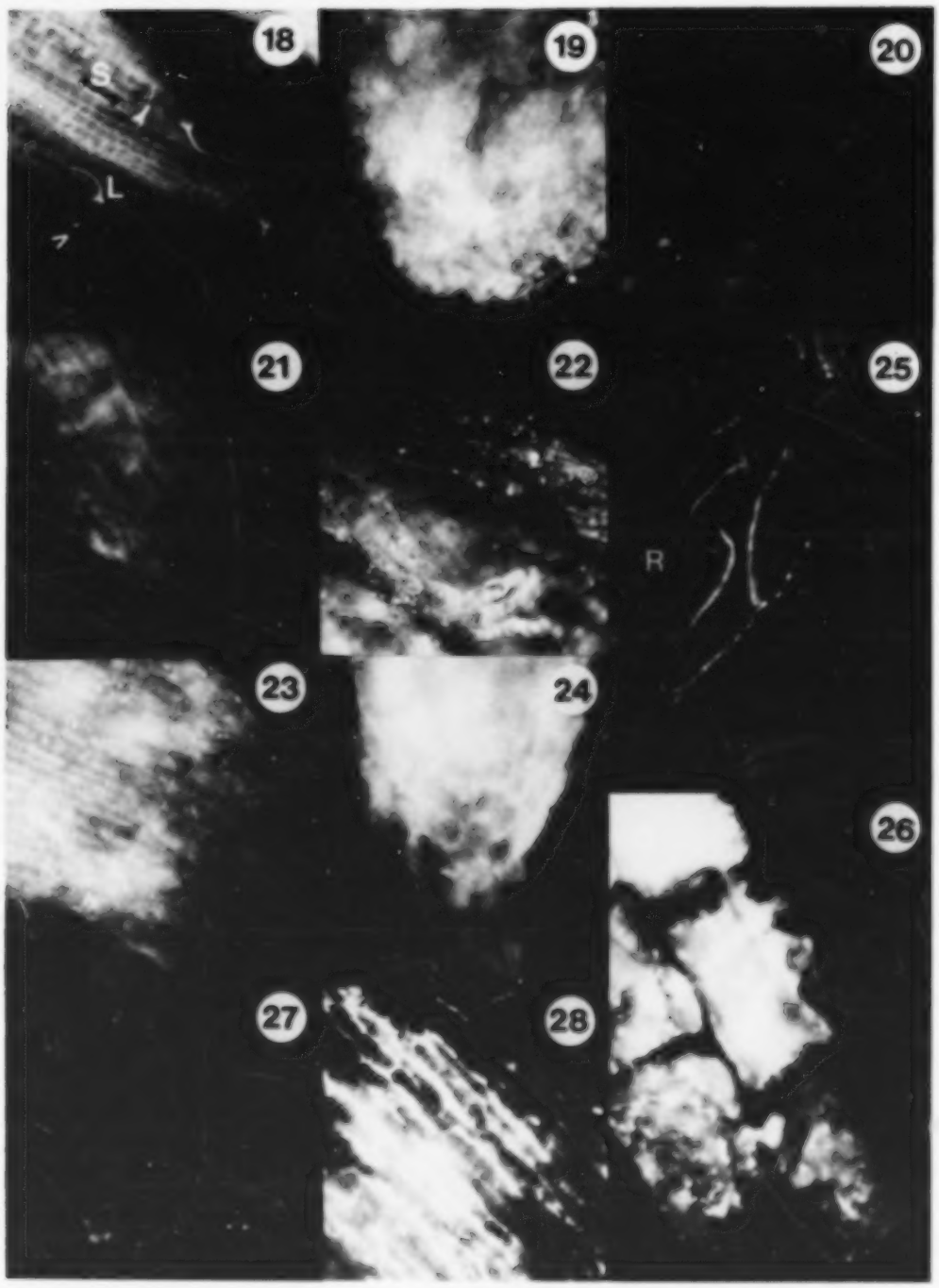
Figure 3-24: Fluorescence micrograph of 'Scout-66' root squash at 400 μM Al; 170x. Note the high fluorescence as compared to Figure 3-23.

Figure 3-25: Fluorescence micrograph of root hairs of 'Scout-66' root squash at 400 μM Al; 110x. Note the callose deposits occurring along the length of the root hair (R).

Figure 3-26: Fluorescence micrograph of 'Atlas-66' root squash at 0 μM Al plus DDG; 685x.

Figure 3-27: Fluorescence micrograph of cortical cells of 'Atlas-66' root squash at 0 μM Al plus DDG, (15 seconds); 110x. Note the numerous callose deposits as compared to control in Figure 3-21.

Figure 3-28: Fluorescence micrograph of 'Atlas-66' root squash at 400 μM Al plus DDG, (20 seconds); 170x. Note the numerous callose deposits as compared to DDG-free control, Figure 3-22.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3.4. Discussion

The effects of Al on root and leaf lengths of the four wheat lines were similar to those reported in previous studies (Briggs *et al.* 1989, Chapter 2), with 'Katepwa' and 'Scout-66' being more sensitive to Al than 'Atlas-66' and PT741. In order to facilitate comparisons with other studies, this callose localization study focused on the international standards of Al-resistance and Al-sensitivity, 'Atlas-66' and 'Scout-66', respectively. Aluminum treatment was associated with decreased organ lengths, and increased callose deposition in both embedded and squashed roots, especially in Al-sensitive 'Scout-66'. This line had the largest decreases in root length (57%) and leaf length (37%), and the largest deposits in both its embedded and squashed roots (Figures 3-9 and 3-24, respectively). As found in Chapter 2, there was little change in the amount of callose in the leaves after Al treatment. Oat mesophyll cell protoplasts, however, synthesize callose after Al treatment (Schaeffer & Walton 1990), and some callose was observed in the squashed tissues (Figure 3-18) thus leaf cells are capable of synthesizing callose. The lack of callose deposition in leaves suggested that little Al-induced injury occurred at the leaf membrane.

Many of the trends found in the callose quantification study in Chapter 2 (Table 2-1, Figure 2-3) were also observed here. Firstly, Al treatment was associated with an increase in callose deposits in 'Atlas-66' root squashes (Figure 3-22 *versus* control 3-21), although no difference was observed in its embedded roots (Figure 3-6 *versus* control 3-5). Secondly, Al treatment was associated with an increase in callose deposits in 'Scout-66' roots in both the embedded (Figure 3-9 *versus* control 3-8) and the squashed tissues (Figure 3-24 *versus* control 3-23), although this was more pronounced in the embedded roots. And finally, Al treatment was associated with a greater increase in callose deposits in the roots of 'Scout-66' than the roots of 'Atlas-66' in both the embedded (Figure 3-9 *versus* 3-6) and the squashed tissues (Figure 3-24 *versus* 3-22).

Although the observations of callose localization generally fitted the results of Chapter 2, deviations were observed for several reasons. Firstly, the amount of callose deposits observed could not be accurately estimated by fluorescence microscopy since the human eye is poor at discerning slight differences in colour and fluorescence (Smith & McCully 1978). Secondly, the results of Chapter 2 were determined from the whole root whereas observations in this study were only of the root tip. Callose was likely unevenly distributed along the root since Al preferentially enters the root at the apex (Polle *et al.* 1978, Rincon & Gonzales 1992). Callose deposits at the root apex would be averaged over the whole root in the callose content determinations, hence callose content would be lower than the observed callose. And finally, the results were affected by how the tissues were fixed. Squashed tissues generally had a greater fluorescence than the embedded tissues, even without Al treatment. For example, a squash of Al-free 'Scout-66' roots (Figure 3-23) showed several deposits, although none were evident in the freeze-substituted tissue (Figure 3-8). This difference likely reflected the rapidity of callose deposition during handling and tissue fixation (Galway & McCully 1987).

Even the best conventional chemical fixatives, including glutaraldehyde and acrolein, allow callose deposition to occur (Hughes & Gunning 1980, Galway & McCully 1987). Squashed tissues were fixed in alcohol which is a good fixative, however, it takes time to penetrate tissue (Jensen 1962). Embedded tissues, on the other hand, were fixed by freeze-substitution which is the most accepted technique for fixing tissues for callose observation (Galway & McCully 1987, Hughes & Gunning 1980). As evident in this study, tissues must be fixed by at least two methods as artefacts due to the technique of preparation would recur until the method was changed (Jensen 1962).

Several other studies found that Al induces callose synthesis in roots (Wissemeier *et al.* 1987, Jorns *et al.* 1991, Chapter 2). Further, the distribution of Al in

roots (Polle *et al.* 1978, Rincon & Gonzales 1992) fitted the distribution of callose along the root periphery (Wissemeier *et al.* 1987, Jorns *et al.* 1991, and Figure 3-9). Hematoxylin staining studies using the Al-sensitive wheat cv. 'Brevor' indicate that Al is localized mostly at the outer layers of root cells, and not near the root tip, unless exposed to very high Al levels (Polle *et al.* 1978). According to Polle, the deposits observed at the root tip of 'Scout-66' (Figure 3-9) were due to the relatively high treatments of 400 μM Al. Rincon & Gonzales (1992) found that 'Atlas-66' roots had much less Al in the root meristem than Al-sensitive 'Tam 105'. These studies suggest that some form of barrier prevents Al from entering the inner cortex of 'Scout-66'; Al resistance in wheat may depend upon a metabolism-dependent exclusion of Al from the sensitive meristems (Rincon & Gonzales 1992, Zhang & Taylor 1989).

This barrier to Al passage might also be associated with observations of a 'gap' in the callose deposits in the cortex of a non-median, longitudinal section of Al-sensitive 'Scout-66' (G, Figure 3-14). This 'gap' appeared to be in the central cap region and, perhaps, the same low Al area that Polle *et al.* (1978) described. This suggestion is clearly speculative as the exact position of these cells cannot be determined from Polle's descriptions. Alternatively, the 'gap' in callose might be due to a resistance mechanism that prevented either callose deposition. Further, encrusting substances, such as lignin or polyphenols, located in this 'gap' might have prevented aniline blue staining (Beckman *et al.* 1982).

Another suggestion for the lack of callose in this region might be related to recent studies of calcium availability - the prerequisite for callose deposition. Aluminum inhibited calcium influx in *Amaranthus tricolor* (Rengel & Elliott 1992) and wheat (Huang *et al.* 1992). The results of Huang *et al.* (1992) were particularly relevant to this study as wheat of similar varieties and ages were used. Using Ca^{2+} selective microelectrodes, Huang *et al.* (1992) found that Al rapidly inhibited calcium ion influx from outer root apical cells (located 2 mm from tip) in Al-sensitive 'Scout-66' but not in

Al-resistant 'Atlas-66'. It is tempting to speculate that the 'gap' observed in Figure 3-14 corresponded to a region of reduced calcium ion influx, as reported by Huang *et al.* (1992), or, alternatively, to a region of low Al, as reported by Polle *et al.* (1978).

Ryan *et al.* (1992) state that because the calcium measurements approached the limit of detection of the system, no conclusions can be drawn regarding calcium flux. Nevertheless even if the study of Huang *et al.* (1992) gained further experimental support, these results might be reconciled. Firstly, if calcium flux were to vary along the length of the root, both spatially and temporally (Huang *et al.* 1992), this might make calcium available for callose deposition in some regions and be undetected in others. Secondly, calcium might not be detected if it were bound to another compound, such as callose synthase, or if it were compartmentalized in organelles (Kauss 1990). Alternatively, callose might have been mistaken, in this study, for another compound with similar fluorescing properties. This, however, seemed unlikely given the results of the DDG and callose hydrolase studies, and the callose quantification study in Chapter 2.

There were several reasons to believe that callose was observed in this study. Although a few compounds have a similar staining property as callose, interference by these compounds was reduced by prestaining tissues with periodic acid-Schiff (Smith & McCully 1978). Autofluorescence of embedded sections was reduced, and callose specificity was improved, by using a concentrated preparation of the active ingredient in aniline blue, Sirofluor (Stone 1984). Aniline blue contains a mixture of compounds having a low and variable fluorochrome concentration (Smith & McCully 1978). Further, callose hydrolase (EC 3.2.1.21) was used on the embedded tissues to test for the β-1,3 glucose linkages of callose. Although not specific for β-1,3 linkages, this enzyme reduced the fluorescence of the tissue (Figure 3-12 *versus* control 3-11), without noticeably affecting the staining of cellulose and other wall components. These factors all suggest that callose was present in the fluorescing deposits.

The final measure to confirm callose presence was by adding the callose inhibitor, DDG, to the nutrient solution of selected plants. Less callose was observed in the embedded sections after DDG treatment (Figure 3-13 *versus* control 3-11). These results resembled those of previous studies involving callose and DDG (Jaffe & Leopold 1984, Bayles *et al.* 1990, Table 2-2 in Chapter 2), and, again, suggested that callose was present; caution must be used in interpreting this study due to lack of replication. Conflicting results were obtained in the root squashes, however, since DDG treatment was associated with an increase in fluorescence in 'Atlas-66' (Figure 3-27 *versus* control 3-21) and 'Scout-66' (not shown). Due to previous studies advocating freeze-substitution for the study of callose (Galway & McCully 1987, Hughes & Gunning 1980), and the results of Chapter 2, it was believed that observations of the embedded roots more accurately reflected DDG action on callose than those of the squashed roots.

In addition to decreasing root callose content, DDG affected the whole plant. Although the action of this compound is not well-known, DDG is likely metabolized into UDP-deoxyglucose and GDP-deoxyglucose (Datema & Schwarz 1979). In viruses and algae, these metabolized forms inhibit first biosynthesis of lipid-linked oligosaccharides, then protein glycosylation - the transfer of completed lipid-oligosaccharides to the protein (Datema & Schwarz 1979, Datema *et al.* 1983). This compound inhibits gravitropism and tendril coiling in *Pisum sativum*, and callose deposition in *P. sativum* (Jaffe & Leopold 1984) and *Phaseolus* stems (Jaffe *et al.* 1985). Such a general action might explain the observed toxic responses of DDG on wheat seedlings, including reduced organ lengths and root damage. Similar responses were reported in Chapter 2. However, no toxic effects were reported by Jaffe & Leopold (1984) after 10 µM DDG was applied to the roots of *Pisum sativum* and *Zea mays*; and, up to 10^{-2} M DDG was not "excessively toxic" to leaf abscission in *Citrus sinensis* (Jaffe & Goren 1988). Fewer toxic effects might have occurred if wheat seedlings were treated

with DDG for shorter periods of time. Without additional knowledge of how DDG affects plants, it is difficult to speculate how the observed toxic responses impact on these results, and further research is required.

In summary, callose was not positively associated with Al resistance. However, the pattern of callose deposition suggested that toxic species of Al penetrated at least the outer portion of the root in Al-sensitive lines, but not Al-resistant lines. This implied that a resistance mechanism may be operating in the outer region of the root. There was no evidence to indicate that toxic species of Al were transported to the leaves. Plant tissues prepared for callose study should be first fixed by freeze-substitution to prevent callose artefacts due to handling and chemical fixation. Further study will be needed to explain the apparent discrepancy of callose deposits between freeze-substituted and chemically fixed roots after DDG treatment. This study emphasized the importance of an unwritten 'law' that a microscopic finding cannot be considered as proven until it has been observed by at least two different techniques (Jensen 1962).

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4. General Discussion and Conclusions

What I tell you three times is true.
"The Hunting of the Snark." Lewis Carroll

Aluminum (Al) affects the whole plant, however primary injuries occur in the root. Some of these Al-induced injuries were reviewed in Chapter 1, and it was suggested that the mechanisms of Al resistance in plants may be revealed by studying callose deposition. In theory, callose may feature in both forms of Al resistance: in avoidance by preventing Al from entering the cell, and in tolerance by localizing the Al to a few cells with sealed plasmodesmata. Was callose deposition involved in Al resistance? To address this question, wheat plants were exposed to Al and their root and leaf callose quantified by spectrofluorometry in Chapter 2, and localized by fluorescence microscopy in Chapter 3. This final chapter presents a brief discussion of the general results of Chapters 2 and 3, a summary of the conclusions, and a list of some of the implications of this study.

Root and leaf lengths were used as a measure of the strain of a treatment (Al, DDG, mannose) on whole plants. Growth responses of the four wheat lines were similar to those of previous studies (Briggs *et al.* 1989), with 'Katepwa' and 'Scout-66' being more sensitive to Al than 'Atlas-66' and PT741. For the three parameters measured, root length, leaf length, and callose content, differences in root length occurred at the lower Al treatments. However, root callose content showed the largest differences between Al-resistant and Al-sensitive lines. Leaf length was highly variable, and was therefore a poor marker for Al resistance in seedlings of this size. These results supported the idea that Al primarily affects the root. Measuring growth responses may be appropriate for older wheat plants (Briggs *et al.* 1989) where plants of uniform size and vigour are available, but this requires more time and labour in the growth chamber than the present study. Further, since growth responses to Al are

among the last events in the perception/transduction/response chain, they may not be the best screen for Al-resistance (Bennet & Breen 1991).

Neither the studies of callose content nor callose localization provided supportive evidence for a role of callose in Al resistance, rather, Al-sensitive lines deposited more callose in their roots after Al treatment. Since callose is deposited in response to injury at the membrane (Kauss 1990), this suggested that greater damage was occurring to the root membrane of Al-sensitive lines than to that of Al-resistant lines. Lines were clearly differentiated by both their callose content and their callose localization. Callose in the embedded tissues of Al-sensitive lines was localized mainly at the root periphery, and did not appreciably occur in Al-resistant lines. Although more callose was observed in the squashes than in the embedded tissues, a similar pattern was observed. Small deposits of leaf callose were also observed in the squashes but not in embedded tissues. Further, after DDG treatment, fewer deposits were observed in embedded tissues than in squashed tissues. As with the embedded tissues, root callose content declined after DDG treatment.

These studies suggested that toxic species of Al may penetrate further into the roots of Al sensitive lines than Al-resistant lines, and that a resistance mechanism may be operating at the root level since no damage (callose deposits) was observed or detected beyond the root tip. Zhang & Taylor (1989) hypothesized that Al resistance in wheat is due to metabolism-dependent exclusion of Al from the sensitive meristems. Wagatsuma (1983) found that the membrane may be an important barrier to the passive movement of Al. It was, however, possible that an Al resistance mechanism prevented toxic species of Al from inducing callose. Clearly, knowledge of the movement of Al is critical. Although a radioisotope for Al is available, it is costly, dangerous, and difficult to acquire. Further, neither radioisotopes nor hematoxylin staining, reveal anything about the toxic nature of the Al species. This is one of the

advantages of using a biological marker such as callose deposition to follow the movement of Al.

There are several difficulties using callose deposition as a membrane marker. Firstly, the sensitivity and rapidity of both callose deposition and degradation complicates interpreting results. These concerns may be alleviated by fixing tissues by freeze-substitution and performing time-course studies of deposition. Secondly, the questionable specificity of aniline blue for callose may overestimate the number of deposits unless more specific dyes, such as Sirofluor, or callose-specific antibodies (Northcote *et al.* 1989) are used. Thirdly, the presence of encrusting compounds such as lignin may impede the detection of callose.

In summary, the following conclusions may be drawn from the experiments described in this thesis: (i) leaf length was a poor indicator of Al resistance in seedlings, (ii) root length was more sensitive to Al than root callose content, but differences between Al-resistant and Al-sensitive lines were not as large, (iii) root callose content was a useful screen for (lack of) Al resistance, (iv) freeze-substitution was the most appropriate means of localizing callose deposits, (v) Al-sensitive lines had more injury at the periphery of the roots than Al-resistant lines, (vi) DDG generally inhibited callose-induced fluorescence, but was also toxic to the plant, and (vii) callose hydrolase reduced callose-induced fluorescence.

This research began with the hopes of finding evidence for a physiological role of callose. In particular, it was thought that callose may feature in Al resistance by blocking plasmodesmata or binding apoplastic Al. Although Al treatment did affect callose content and localization, callose was negatively correlated with Al resistance in wheat. Nevertheless, these studies have several potential applications. Root callose content may be used as a non-destructive screen of wheat genotypes for Al resistance. This is 'non-destructive' since only the root tips of the plant are harvested; the rest of the plant remains intact and available for crossing. This would benefit plant breeders

by reducing the time and labor required for screening metal resistant wheat cultivars. Root callose content studies are rapid, sensitive, and possibly applicable to a variety of stresses, plant species and plant growth systems (solution culture and cell suspension).

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